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GENOTOXICITY AND CARCINOGENICITY

Revealing DNA damage levels in rat testicular germ cells in vivo using an adapted version of the alkaline comet assay

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Abstract

Heritable mutations in male germ cells pose a critical risk to human health and future generations, however standardized methods for assessing germ cell genotoxicity remain limited. We refined the in vivo alkaline comet assay (proof-of-concept (Dirven et al. 2023); protocol (Olsen et al. 2024)) to detect DNA damage in testicular germ cells, with selective addressment of haploid spermatids and primary spermatocytes. Measurements of DNA damage (% Tail DNA) and DNA content (total fluorescence intensity) in individual comets were combined with visual comet identification to distinguish testicular comet populations based on differences in DNA content and appearance. To verify the method's functionality and reliability, DNA damage was assessed in rats exposed to the direct-acting, well-characterized genotoxicants X-rays and ethyl methanesulfonate across distinct testicular cell populations, alongside liver and blood. To minimize experimental variation, the protocol included stringent standardization of animal handling, tissue processing, and comet assay procedures. Both X-rays and EMS induced significant DNA damage in testicular germ cells, with comparable responses across testicular cell types and similar (X-rays) or higher levels observed in somatic tissues. The low inter-animal variability observed supports the robustness of the method. Importantly, inclusion of testicular germ cells in OECD test guideline 489 would provide a valuable tool for hazard identification and mutagenicity classification of chemicals under the Globally Harmonized System of Classification and Labelling of Chemicals. This versatile, sensitive, and resource-efficient assay enhances the assessment of male-mediated genetic risks and supports regulatory efforts to protect reproductive health and safeguard the genetic integrity of future generations through the use of safer chemicals.

Keywords OECD TG 489 · Comet · Genotoxicity · Testis · Germ cell · Spermatid · Primary spermatocyte

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Introduction

Heritable mutations transmitted via the male germline are a major route for genetic change transmitted to future generations and affect disease risk, including childhood cancer (Beal et al. 2017; Bonde et al. 2019). DNA sequence alterations, including point mutations, insertions, deletions, and structural rearrangements, are permanent and commonly arise during spermatogenesis from DNA damage, replication errors, error-prone or missing DNA repair. Both endogenous biological processes and exogenous environmental exposures can lead to such genetic changes. Paternal mutations can be incorporated into the zygote or fixed after fertilization and can persist across generations unless removed by negative selection (Kaplanis et al. 2022). Therefore, there is a need to assess risks of male-mediated transmission of harmful effects to future generations following environmental stressors. However, few standardized, validated test methods are currently available.

During spermatogenesis, germ cells develop into mature spermatozoa, undergoing defined changes in ploidy (n) and DNA content (C) (Fig. 1). Diploid spermatogonia (2n/2C) divide by mitosis to maintain the stem cell pool or produce primary spermatocytes (PS). PS are diploid (2n) with duplicated chromatids and hence have double the amount of DNA (4C) and divide to produce secondary spermatocytes (n/2C; Meiosis I), that rapidly undergo meiosis II into haploid (n/1C) spermatids which differentiate into mature spermatozoa. Within the seminiferous tubules, the spermatogonia are located on the basal side of a gradient of increasing protection from exogenous influences (the so-called blood-testis barrier; (Mruk and Cheng 2015; Wanjari and Gopalakrishnan 2024).

Outcomes of germ-cell DNA damage depend on spermatogenic stage, seminiferous-tubule location, repair capacity, and DNA synthesis to fix lesions as mutations. DNA synthesis is restricted to the mitotically active spermatogonia and to DNA repair-associated synthesis. DNA repair capacity in male germ cells varies by both cell stage and damage type (Jansen et al. 2001; Olsen et al. 2001, 2003, 2005). Less is known about DNA repair in spermatogonia located outside the testis barrier, however risk is mitigated through high susceptibility to programmed cell death (Rotgers et al. 2015). DNA damage persisting in the mature spermatozoa can be converted into mutations in offspring, as demonstrated to contribute to the paternal de novo mutation and age effects in disorders such as achondroplasia, Apert syndrome, and some neurodevelopmental conditions (Kong et al. 2012).

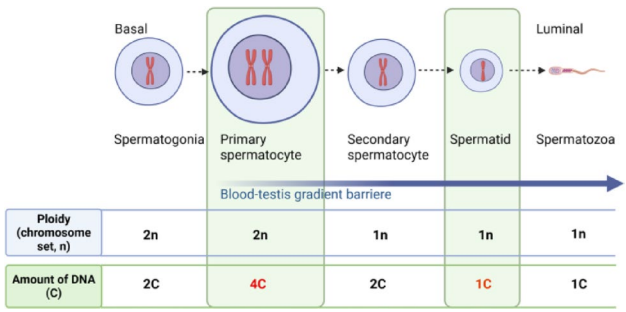

Fig. 1 Spermatogenesis. The figures illustrate the progression from spermatogonia to spermatozoa, showing the number of chromatids in each cell type, including the location of each cell type within the seminiferous tubules (basal or luminal side), the blood-testis barrier gradient, and ploidy and DNA content of each cell type. The green boxes highlight the two germ cell types of particular interest in this study. Created in BioRender. Olsen, A. H. (2026) https://BioRender.com/qpe1snh

Environmental factors can elevate mutation rates in the male germline and are associated with offspring health challenges, including childhood cancer (reviewed in (Beal et al. 2017); (Bonde et al. 2019; Chang 2009; Cordier et al. 2004), congenital malformations, pregnancy loss (Beal et al. 2017), and male infertility. Exposures such as smoking, ionizing radiation, industrial chemicals, heavy metals, and certain chemotherapeutic agents can directly damage DNA in spermatogonial cells, and if unrepaired or mis-repaired lead to heritable mutations (Anderson et al. 2014; Beal et al. 2017; Bonde et al. 2019; Linschooten et al. 2013; Trasler et al. 1985; Wyrobek et al. 2005). For radiation, heritable mutations are well documented in rodents, whereas evidence in humans remains inconclusive (reviewed in (Stephens et al. 2024)). In contrast, simple alkylating agents have been clearly shown to induce paternal transmission of mutations to offspring, across germ cell stages, in rodents decades ago (Generoso et al. 1974). More recently, genome-wide sequencing and mutational signature studies in humans have demonstrated both pre-conceptionally and post-zygotic paternally transmitted mutations from chemotherapeutics (Kaplanis et al. 2022).

To reduce the risk of paternal germline mutations regulation of chemical substances is essential to protect current and future generations. For hazard identification of environmental exposures, internationally accepted, validated and standardized protocols are needed. Such methods are developed and described by The Organisation for Economic Co-operation and Development (OECD). However, standardized assays for germ cell mutagenicity are scarce and often animal-intensive, limiting hazard assessment. The comet assay is a powerful tool for measuring DNA damage as single-strand breaks (Collins 2004; Olive and Banáth 2006; Ostling and Johanson 1984; Singh et al. 1988), and has been shown to provide data from testicular germ cells (Asare et al. 2016, 2012; Bjorge et al. 1996a, 1995, 1996b; Brunborg et al. 2015; Collins 2004; Graupner et al. 2017, 2015, 2014; Gutzkow et al. 2016; Hansen et al. 2014,

2010; Olive and Banáth 2006; Olsen et al. 2001, 2003, 2005; Sharma et al. 2018; Speit and Hartmann 2006; Speit et al. 2009; Tice et al. 2000), also in a regulatory context (Brendler-Schwaab et al. 2005; Frotschl 2015; Graupner et al. 2014; Koppen et al. 2017; Vasquez 2012). The OECD TG 489 In vivo mammalian alkaline comet assay (TG 489; (TG489 2016)) indicator test has been validated for somatic cells (liver and stomach), however testing in testicular germ cells was not recommended without further validation, due to the presence of somatic cells in testicular cell suspensions. We therefore developed a comet assay protocol that selectively assesses the DNA damage in the germ cells of the testis (Dirven et al. 2023; Olsen et al. 2024), addressing the limitations outlined in TG 489. Moreover, obtaining supportive data directly from the relevant target cell type, i.e., germ cells, is highly relevant for meeting the classification requirements of the Globally Harmonized System of Classification and Labelling of Chemicals (GHS 2021) and the CLP Regulation (Classification, Labelling, and Packaging; Regulation (EC) No 1272/2008 (CLP 2008)) for the Mutagenicity classification.

In this study, we evaluated the applicability of the testicular germ cell comet protocol developed using two well-characterized direct-acting genotoxicants (X-ray and Ethyl methanesulfonate (EMS)) in rats. The aim was to determine whether this germ cell–targeted comet assay could serve as a robust tool for regulatory toxicology and basic research. DNA damage levels were analyzed in different germ cell types, including 1n/1C haploid spermatids and 2n/4C PS, as well as in > 1C testicular cells (germ and somatic cells) and somatic tissues (liver and blood). Additionally, DNA damage across testicular and somatic cell types, and the effects of different EMS exposure scenarios, and variability in control samples were analyzed.

Materials and methods

Materials

Chemicals for exposure of animals were obtained from Merck (Søborg, Denmark) and included Ethyl methanesulfonate (EMS; CAS # 62-50-0, catalog number not provided). Saline (0.85%) CAS #: 7647-14-5. For exposure to X-rays, an RS 2000 Quastar 2 irradiator (Rad Source Technologies Inc, 4907 Buford, Georgia, US) was used. Identical reagent batches and preparations of solutions and buffers were used throughout the experiments.

Animals

The animal study was conducted under conditions approved by The Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee. Male Wistar Han IGS rats, 11 weeks of age were purchased from Scanbur (Karlslunde, Denmark). Animals were allowed to acclimate for one week, weighed, and randomly divided into nine dose groups (Table 1). Each group consisted of 5–6 animals according to the recommendations in TG 489 (TG489 2016). The rats were housed individually in cages (1291H Eurostandard type III H, Techniplast Gazzada, Buguggiate, Italy) with wood bedding (Tapvei, Paekna, Estonia) under controlled conditions (temperature of 22 ± 1 °C, relative humidity of 55 ± 5%, 12 h light/dark cycle, air change 75 times/h). Food (Safe A-30, Germany) and tap water were given ad libitum. During the acclimation and study periods, the rats were observed twice daily for changes in appearance or behavior.

Table 1 Animals and exposure

Agent, exposure	Number of animals	Weight mean ± SD (g) at randomization	Exposure conditions	Day(s) of exposure	Day of necroscopy
X-rays, Gy			**Irradiation time (1.2 Gy/min)**		
Sham[a]	5	330.8 ± 22.2	6 min 40 s	Day 0	Day 0[c]
4 Gy	6	331.5 ± 13.1	3 min 20 s	0	0[c]
8 Gy	6	330.5 ± 11.7	6 min 40 s	0	0[c]
Ethyl methanesulfonate (EMS) - 200 mg/kg[b]			Dose		
2 days solvent	5	331.4 ± 18.2	Saline X 2	0, 1	1[d]
2 days exposure	6	331.0 ± 8.6	EMS X 2	0, 1	1[d]
3 days solvent	5	331.2 ± 18.5	Saline X 3	0, 1 ,2	2[d]
3 days exposure	6	330.8 ± 18.6	EMS X 3	0, 1, 2	2[d]
4 days solvent	5	300.0 ± 10.1	Saline X 4	0, 1, 2, 3	3[d]
4 days exposure	6	331.3 ± 17.0	EMS X 4	0, 1, 2, 3	3[d]

[a]The X-ray control (sham) animals were placed in the irradiation chamber for 6 min and 40 s without irradiation

[b]Animals given EMS were exposed two, three or four times with 24 h between each dose

[c]Immediate necroscopy

[d]Necroscopy 3 h after last dose

Exposure

Two well-known genotoxicants, X-rays and EMS, were studied. The number of rats per group was 5 for the control groups and 6 for the treated groups (Table 1). To expose to X-rays, rats were placed in a narrow, hard plastic container to restrain them during exposure. The rats were exposed in random order, irrespective of exposure group. The box was placed in a cage and put into the irradiator. Whole-body irradiation was given with 1.2 Gy X-rays/min. The treatment groups were exposed to total doses of 4 (3 min 20 s) and 8 (6 min 40 s) Gy, whereas the control group animals were placed in the irradiator for a similar length of time (6 min 40 s) as the 8 Gy group. After irradiation, the rats were immediately terminated. In the EMS study, the rats were weighed before each dosing. EMS was diluted in 0.85% saline. Each day, fresh EMS-solutions were prepared (20 mg/mL), and administered by oral gavage at 10 mL/kg bw, in a group-by-group manner. Groups of rats received EMS or saline 2, 3 or 4 times, with 24 h intervals between doses, and with separate solvent control groups for each dose regimen (2, 3 or 4 daily exposures; for details see Table 1). Animals exposed to EMS or saline were terminated exactly 3 h after the last exposure, in accordance with the sampling times recommended in TG 489 (TG489 2016). To standardize the procedure, a strict fixed time (10 min) was followed between each animal for dosing, termination, and all further processing in all groups (Fig. 1).

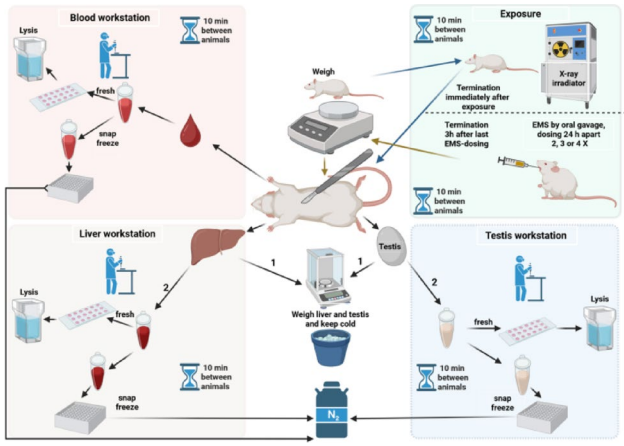

Fig. 2 Illustration of the experimental workflow with separate workstations for each operation. We used strict time intervals of 10 min between animals for all operations. One trained operator was dedicated to each workstation for (1) exposure (X-rays or EMS), (2) weighing, necropsy and blood extraction, (3) dissection and weighing of tissues, and (4–6) single-cell preparation of each tissue. Necropsy occurred immediately after X-rays and 3 h after the last dosing of EMS, and all subsequent operations were kept cold. After necroscopy, blood was drawn immediately, and rapidly thereafter, the testis and liver were excised, chilled and weighed. Single-cell preparations from each tissue were immediately prepared at the corresponding workstations. Cells from one cell aliquot was embedded in agarose on Gelbond® films and submerged in lysis solution for comet assay. Other aliquots were snap frozen. Created in BioRender. Olsen, A. H. (2026) https://BioRender.com/ww5g4bm

Animal termination, tissue harvest and preparations of single cell suspensions

Technical standardization was attempted by dividing the workflow into workstations operated by the same trained dedicated operator throughout the experiments (Fig. 2). This was done to obtain consistent processing and timing of animal termination, ensuring uniform and rapid dissection and processing of biomaterials. An overview of the workflow for animal termination, tissue harvest and cell preparation is illustrated (Fig. 2**)** with one workstation for animal exposure, one for animal termination, one for dissection and weighing of tissues and separate workstations for each biomaterial (blood, testis, liver). Animals exposed to EMS were weighed before termination by first rendering them unconscious due to a blow to the skull, followed by immediate decapitation. Blood was collected immediately after decapitation from the neck veins into an EDTA microvette tube and mixed gently. At the next workstation, the abdomen was opened, and the left testis was excised and instantly submerged in PBS on ice to cool. The left testis was blotted to remove excess liquid, and weighed, and was returned to the PBS on ice for immediate preparation of single-cell suspensions (scs) at the Testis workstation. The left lateral lobe of the liver was excised, weighed and rapidly chilled in PBS on ice for immediate preparation of liver scs at the Liver workstation. The remaining liver was harvested and weighed. The relative organ weights were calculated as organ weight divided by animal body weight at termination.

Following collection of blood it was immediately transferred to the Blood workstation (Fig. 2) where 100 µl blood was transferred to a pre-aliquoted tube containing 500 µl (50% Merchants solution with 10 mM EDTA, 40% fetal bovine serum, 10% DMSO (M/F/D)) buffer as previously described (Olsen et al. 2024) using a wide-bore tip and placed on ice. The blood was aliquoted into 30 µl portions and snap-frozen in liquid N_2, leaving one aliquot on ice for comet analyses.

The testis and liver were prepared as described (Olsen et al. 2024). The testis was decapsulated in a Petri dish on a cold steel plate by making incisions in the capsule and gently squeezing out the seminiferous tubules and removing the main blood vessel. Using tweezers and a scalpel, the testis was cut into small pieces where all except one piece were transferred to pre-labelled tubes and snap-frozen in liquid N_2. The testis piece was submerged in 1 mL M/F/D-buffer and testicular cells were gently squeezed out of the seminiferous tubules using several strokes with a triangular

bacterial spreader. The cells were gently homogenized by pipetting and filtered through a sandwich of gauze on top of a 100 µM nylon membrane filter into a collection tube, on ice. The cell sample was diluted 1:50 with M/F/D-buffer and rapidly counted manually in a Bürker chamber using a phase-contrast microscope without staining the cells. The scs was adjusted to obtain a cell concentration suitable for comet analyses (~0.8–1 × 10^6/mL), while kept on ice. Aliquots of diluted testis scs (30 µl) were snap frozen in liquid N_2, leaving one aliquot on ice for the comet analyses.

The liver lobe was cut into small pieces using a scalpel in a Petri dish on a cold steel plate. One piece was used to prepare liver scs using a method developed by Brunborg and co-workers (Brunborg et al. 1990) where the tissue piece is forced through an ice-cold metal sieve (15 mm internal diameter, stainless-steel screen with mesh size 0.4 mm) submerged in 2 mL M/F/D-buffer on ice using 2 strokes with a plastic plunger, and filtered through a 100 uM nylon membrane filter into a collection tube on ice. The samples were diluted 1:30 with M/F/D-buffer, and the cell concentration was rapidly counted, similarly as the testis scs, and the cell concentrations were adjusted to ~0.8–1 × 10^6/mL. The final dilution was aliquoted (30 µl portions), and snap frozen, leaving one aliquot on ice for the comet analyses.

Alkaline single-cell gel electrophoresis (SCGE)

The alkaline SCGE assay was performed according to the protocol described (Graupner et al. 2017, 2016, 2014; Gutzkow et al. 2013) with minor adaptations. One workstation was dedicated to the embedding of cells and lysis. A single trained operator embedded the single-cell preparations for each tissue. Prelabeled Gelbond® films were placed on a cold steel plate. The scs were kept on ice until embedded in low-melting-point (LMP) agarose on Gelbond® films. All cell types from a given animal were embedded on the same Gelbond® film. To embed cells in gels, an automated flexible pipette was used to mix 10 µL of scs with 90 µL of 0.75% LMP (final concentration 0.675%) agarose, and immediately 4 µL aliquots of the mixed scs/LMP agarose for each replicate were pipetted on the Gelbond® film and allowed to solidify. All cell types (testis, liver and blood) from 1–2 animals were embedded on each Gelbond® film, with 8 gels for testis, liver, blood and blood/testis-mix per animal. The films were carefully submerged in cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate with pH 10 at 4 °C, added 1% Triton X-100 and 10% DMSO on the day of the experiment) where it was kept during transport from DTU, Denmark to NIPH, Norway for conduction of the subsequent steps of the comet protocol. For the Gelbond® film setup, each electrophoresis chamber fits 4 Gelbond® films and 2 chambers can be run simultaneously, connected to the same power supply. The 17 X-ray animals were placed on 9 films, with films 1–8 in one run and film 9 (one 4 Gy animal) in a separate run on the same day using the same electrophoresis solution. Animals given 2, 3 or 4 daily EMS or saline doses (11 animals per exposure scenario) were placed on 6 films and run as one single run per experimental regimen, and on consecutive days to obtain similar time conditions for each exposure and termination regime. The films were rinsed rapidly in distilled water and transferred to a tank with fresh cold alkaline electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13.2) and left for 40 min at 4 °C to unwind the DNA. Before running the electrophoresis, the tanks were filled with freshly prepared electrophoresis solution, the power supply voltage was set to 25 V. The voltage was measured across the stage using a voltmeter and was adjusted to a V/cm of 0.86 (range 0.84–0.88) by adjusting the volume of electrophoresis solution. To further ensure uniform electrophoresis conditions, the electrophoresis solution was kept cold and circulated throughout electrophoresis. The films were briefly rinsed in distilled water for 1 min and neutralized by submerging in PBS twice for 5 min. The films were briefly rinsed with distilled water twice and submerged in absolute ethanol with minimal carryover of water residue for 5 min. Next, the films were fixed for > 90 min in fresh absolute ethanol and air-dried overnight. Before staining the DNA, the light was dimmed to prevent fading of the SYBR™ Gold Nucleic Acid Gel Stain. The films were submerged in a box containing fresh 1 X SYBR™ Gold Nucleic Acid Gel Stain diluted in TE-buffer (10 mM Tris–HCl, 1 mM Na_2EDTA pH 8.0) for 20 min with gentle rocking. Subsequently, the films were rinsed in distilled water for 1 min and placed on top of wetted paper towels in an enclosed light-proof box to create a humidified atmosphere, and stored at 4 °C. Stained films were covered with large coverslips (80 × 120 mm, thickness no. 1; VWR International AS, Oslo, Norway) and scored within few days.

Comet visualization and analysis (scoring)

We followed our protocol (Olsen et al. 2024) with some adaptations. All comets were scored blindly and by the same operator throughout the experiments. To score comets the Gelbond® films were placed on the stage of a fluorescence microscope mounted with video camera (Olympus BX51 microscope with Olympus BH2-RFL-T3 light source and A312f-VIS CCD camera), with the Comet IV analysis software (Perceptive Instruments Ltd) for semi-automated scoring. Gels with suitable densities of comets were selected among the 8 gels per sample to prevent scoring bias due to high comet density, and the operator scored comets in a systematic pattern across each gel, avoiding the gel edges. For

the testicular comets from each animal, each gel was scored twice. The first round of scoring was for the selection of 1C comets and the second was for scoring 4C PS. In the first round of scoring all comets with round-shaped comet heads were scored ("all round comets") and served as the basis for the selection of 1C comets. Elongating/elongated spermatids and spermatozoa, identified based on their distinct chromatin structure features, were not scored. The elongating/elongated spermatid comets appear as crescent-like chromatin structures, whereas comets of spermatozoa are curved, small, yet highly condensed chromatin structures. We aimed at scoring > 500 round comets per animal. During the first scoring, the operator noted the high total intensities (DNA content) of the particularly large, and thus easily identifiable, 4C PS comets. In the second round of scoring, these particularly large comets with high total intensity (DNA content) levels were selectively scored to obtain a dataset of comets enriched in 4C PS. We aimed at scoring > 300 enriched 4C PS per animal. For liver and blood, 150 comets were scored for each animal.

Selection of testicular germ cell comets

Selection of testicular germ cell comets were performed as described (Olsen et al. 2024) with minor modifications. Scoring was done semi-automatically on blinded samples to limit operator bias. During spermatogenesis, haploid spermatids with a DNA content of 1C are produced. 1C cells do not exist among somatic cells. This feature was exploited to select the germ cells (haploid spermatids) from the mixture of testicular cells. The 1C spermatid comets were selected from "all round comets" scored during the first round of scoring, based on their low DNA content by setting a threshold between 1C comets and > 1C comets. The thresholds were determined in comet Total intensity (i.e. DNA content; Tot_Int) distribution histograms showing the populations of comets in each animal (see example plots (Fig. 3) in Olsen et al. 2024 (Olsen et al. 2024)) and plots from representative animals that are presented in Fig. 3). The comets scored on all gels for each tissue and scoring round for testis were collapsed into one dataset per animal, of which the median % Tail Intensity (% TI, presented as % Tail DNA; DNA damage level) was calculated. The distribution histograms included Tot_int on the X-axis and comet frequency on the y-axis. The protocol describes two approaches for selecting the 1C comets, either by visual/manual determination of the Tot_int threshold between 1C and > 1C comets template displaying the distribution histograms, or by modelling in an R-script (Olsen et al. 2024). Alternatively, an excel template was provided for users not accustomed to R (Olsen

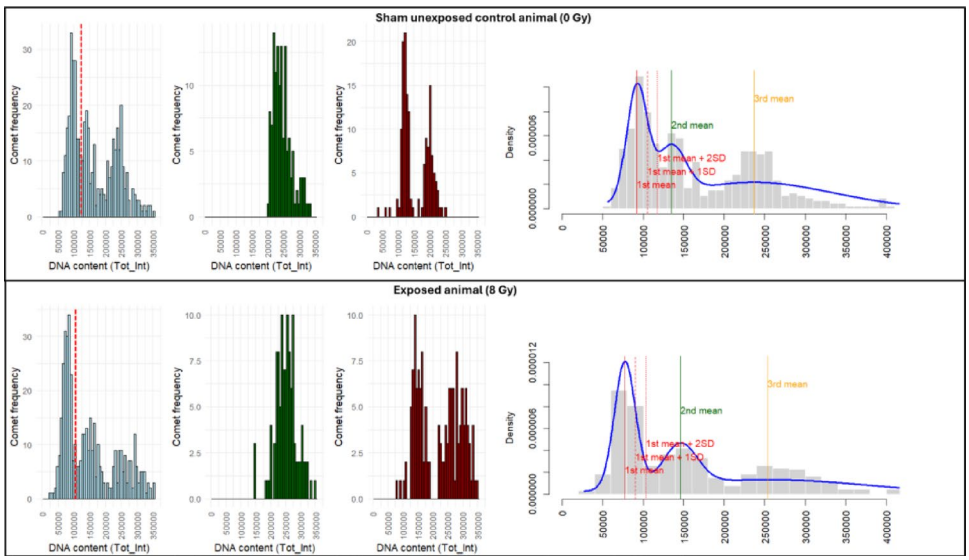

Fig. 3 Histograms of DNA content versus frequency of comets for selection of 1C testicular comets. Results from representative animals are shown in the upper panel (sham animal (0 Gy)) and the lower panel (exposed animal (8 Gy)). The distribution histograms on the left show DNA content (Total intensity; Tot_Int) binned along the X-axes and comet frequencies on the Y-axes. Plots from left to right represent "all round comets" of the testicle (light blue, with the three populations 1C, 2C and 4C), primary spermatocyte comets (green, representing 4C DNA content) and liver (brown, showing the cell cycle with G1 (representing 2C), S and G2 (representing 4C) phases). The red dotted line in the left "all round comet" plot represents the threshold between 1C comets and > 1C comets. The plots on the right show histograms of the testicular "all round comet" comet Tot-Int (X-axes) with a Gaussian mixture model fit (blue) constrained to three populations. The blue line represents the overall fitted distribution, showing the likelihood of observing each value and combining all three modeled populations. The red vertical line represents the mean of the 1C population, with dashed and dotted lines showing mean + 1 SD and mean + 2 SD, as potential threshold values between 1C and > 1C comets. The green vertical line represents the mean of the 2C population, and the orange vertical line represents the mean of the 4C population. For further details see (Olsen et al. 2024)

et al. 2024). The comets below the Tot_int threshold were selected as 1C comets.

Using the developed R-script-based visual/manual approach, a threshold between 1C and > 1C comets was manually identified by inspecting the distribution histograms of the comet Tot_int of each animal, conservatively, to limit inclusion of >1C comets. Using the R-script modelling approach, non-relevant datapoints were first excluded by evaluating the "all round comets" Tot_int distribution histograms of each animal and setting a lower and upper cut-off based on the Tot_int values of all comets. This was done to prevent non-relevant data points from interfering with the modelling-based identification of comet populations. Modelling-based selection of 1C comets was done by modeling the comet Tot_int of each animal using a Gaussian mixture model with forced adaptation to a 3-population model of the comet Tot_int, assuming 3 populations of comets. Using the latter approach, the Tot_int threshold between 1C and > 1C populations was identified as the mean + 1 or 2 SD of the population with the lowest Tot_int (the 1C population). Each plot was inspected to determine the appropriate number of + SD, as a threshold between 1C and > 1C, and to limit > 1C comets from inclusion among the selected 1C comets. Confirmation of correct selection of the 1C comet population was performed by comparing with the Tot_int distribution histograms of PS, liver and blood, plus a sample containing a mix of testicular cells and blood (spike-in), in each animal, since the somatic tissues do not have comets with 1C DNA content. The data presented herein was derived using the visual/manual version of the R-script. For both approaches, the median % Tail DNA of the selected comet populations from all gels scored for each biological replicate (animal) was calculated. The mean % Tail DNA + SD of the medians was derived for each experimental group.

Statistical analyses

Statistical analyses were conducted using Excel, JMP Pro 18 and GraphPad Prism 10. The normality of data distributions was tested using the Shapiro–Wilk test, and the equality of group variances was tested using the Levene test. In cases of non-normality, normalisation of the data by square root, cube root, log2, exponential, and log10 were attempted to achieve data normality and homogeneity. The final statistical testing was done on raw untransformed data. To retain the power of the statistical testing, all data were processed using parametric tests, although some experimental groups did not fully meet the assumptions of normality and homogeneity of variances. The Student T-test (unpaired, two-tailed) was used for comparisons between two groups, whereas ordinary one-way ANOVA followed by Dunnett's (comparison with control) or Tukey's (pairwise comparisons between groups)) post-hoc tests were used for comparisons involving more than two groups. Linear relationships between X-ray exposure and DNA damage levels were investigated by Pearson correlation. Statistical significance was accepted at the 0.05 significance level. To calculate the number of animals required per group with 80% power to detect a twofold difference with $p < 0.05$ was done using the Wilcoxon rank sum power analyses.

Results

The main aim of this study was to demonstrate that the newly developed protocol for DNA damage analyses in testicular germ cells in the in vivo alkaline comet assay successfully retrieves data from specific germ cells of the testis as a useful tool for hazard identification in chemical regulation and to investigate the dynamics of DNA damage induction and removal in vivo. For this purpose, rats were treated with X-rays or EMS.

Experimental standardization, comet scored, and selection of testicular germ cell comets

Experimental procedures were strictly standardized (Fig. 2), including identical reagent batches and solutions, exposure conditions, tissue collection, preparation of single-cell suspensions, embedding in agarose, and subsequent comet assay analysis, including gel electrophoresis and comet scoring. All operations were consistently performed by the same trained operator. To further strengthen consistency, animals were processed at fixed 10 min intervals for dosing, termination, single-cell preparations, and embedding in agarose (Fig. 2).

To robustly identify the 1C comet population, high numbers of testicular "all round comets" (about 500) were scored for each animal to establish a reliable threshold between 1C and > 1C comets since the threshold-setting to separate 1C comets from > 1C comets is based on the distribution histograms of "all round comets" with DNA content (Tot_int) on the x-axes and frequency of comets on the y-axes. Distribution histograms with comets from testis, with "all round comets", primary spermatocyte and liver comets from one representative control (sham, 0 Gy) and one treated (8 Gy X-rays) animal are presented (Fig. 3). The data presented were obtained using the R-script with thresholds between 1C and > 1C comets determined by visual inspection of the distribution histograms, as described in (Olsen et al. 2024). Three comet populations are observed among "all round comets" representing 1C spermatid comets, 2C, and 4C comets. The red dotted line indicates the threshold between

1C and > 1C comets and distinguishes the two comet populations. The distribution histograms of PS (Fig. 3) and somatic comets (liver and blood) were used to confirm the identity of each germ cell population based on the 2C and 4C Tot_int of these cell types. A total of 24,103 "all round comets" were scored for selection of 1C comets (i.e. haploid spermatids) with 159–839 comets in each animal and an average number of 492 comets (Supplementary Material 1). Of these, 24–428 1C comets were selected, with an average percentage of 1C comets of 46% (Range 11–65%; Supplementary Material 1).

Testicular germ cells of 1C comets were successfully selected from the "all round comets" of the testicle when following the approach of the in vivo alkaline electrophoresis method developed to capture testicular germ cell-specific data, as described (Dirven et al. 2023; Olsen et al. 2024). The testicular comet data presented here were obtained by processing the raw comet data using the visual version of the R-script, with manual threshold setting between 1C and > 1C comets.

X-rays

In random order, rats were acutely exposed to 4 and 8 Gy of X-rays, and control sham animals were placed in the irradiator for the same time length as the 8 Gy group. The animals were sampled immediately to capture the induced DNA damage and to systematically restrict the elimination of DNA lesions by DNA repair processes.

The rats were weighed at randomization, before exposure/dosing, and at termination, immediately after exposure (Supplementary Material 2). Due to the immediate termination after exposure, no changes in body weights, absolute or relative testis or liver weights were detected (Supplementary Material 2).

For all tissues and cell types, we observed statistically significant increases in DNA damage levels (% Tail DNA) in exposed animals at both dose levels (4 and 8 Gy) compared with the sham-exposed control animals (Table 2). The mean background control levels ranged from 0.2 to 3.2% Tail DNA, with the lowest levels in 1C comets and blood comets, and the highest in enriched PS. The mean DNA damage levels induced ranged from 9.2 to 15.4% Tail DNA for 4 Gy and 20.2–33.8% Tail DNA for 8 Gy. The levels of DNA damage after 8 Gy were similar in the selected comets of 1C spermatids, in "all round comets", in the enriched PS and liver (range of 20.2–22.6% Tail DNA), whereas the % Tail DNA levels observed in blood cells (33.8% Tail DNA) were significantly higher (One-way ANOVA with Tukey post-hoc test, blood vs. 1C comets ($p = 0.001$); vs. "all round comets" ($p = 0.002$); vs. PS ($p = 0.007$); vs. liver ($p = 0.001$)) than in the other cells. Simple trend analyses (Pearson correlation; Table 2) showed that the DNA damage levels induced by X-rays fit well to linear dose-related relationships in all cell types from the testis and liver (R^2 range 0.82–0.96), with blood cells exhibiting the best fit ($R^2 = 0.96$). The slopes in the different testicular germ cells (1C comets, "all round comets", enriched PS) and liver were highly similar (range 2.4–2.6), with enriched PS showing the smallest slope. Blood displayed almost twofold larger slope (4.2) than the other cell types.

Table 2 DNA damage induced by X-rays in testis, liver and blood

	0 Gy % Tail DNA ± SD	4 Gy % Tail DNA ± SD, *p* value[a]	8 Gy % Tail DNA ± SD, *p* value[a]	Trend analysis by simple linear regression (Pearson correlation)[b]
1C comets	0.2 ± 0.1	9.2 ± 3.1, $p < 0.0050$	20.4 ± 6.1, $p < 0.0001$	Y = 2.538*X − 0.2402 $R^2 = 0.8241$ $p < 0.0001$
«All round comets»	0.5 ± 0.1	10.4 ± 3.2, $p < 0.0019$	21.1 ± 5.9, $p < 0.0001$	Y = 2.574*X + 0.3562 $R^2 = 0.8393$ $p < 0.0001$
Primary spermatocytes	3.2 ± 1.7	15.4 ± 2.8, $p < 0.0001$	22.6 ± 4.9, $p < 0.0001$	Y = 2.405*X + 4.179 $R^2 = 0.8401$ $p < 0.0001$
Blood	0.2 ± 0.05	13.9 ± 2.2, $p < 0.0001$	33.8 ± 3.7, $p < 0.0001$	Y = 4.229*X − 1.008 $R^2 = 0.9611$ $p < 0.0001$
Liver	0.3 ± 0.2	9.8 ± 3.3, $p < 0.0006$	20.2 ± 4.5, $p < 0.0001$	Y = 2.493*X + 0.1418 $R^2 = 0.8750$ $p < 0.0001$

[a] *P* values express statistical significance of the mean % Tail DNA of treated animals compared to sham animals using one-way ANOVA and Dunnett's post hoc test

[b] Trend analyses using simple linear regression by Pearson correlation

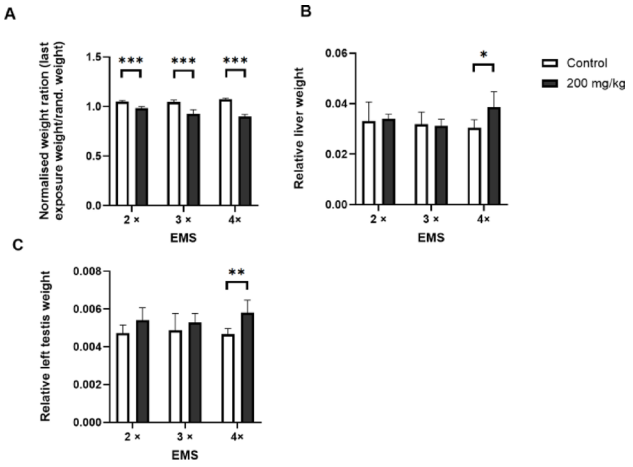

Fig. 4 Animal and organ weights. Normalised body weight ratios are presented in **A**, where the normalized body weight was calculated as the body weight at the last day of exposure divided by the body weight at randomization. Relative liver and left testis weights are presented in **B** and **C**, respectively. The x-axes represent the EMS exposure scenarios with two (2X), three (3X) or four (4X) exposures with 24 h intervals. Statistical significance was tested using the unpaired two-tailed Student's T-test, with significance levels *$p<0.05$, **$p<0.01$, ***$p<0.001$

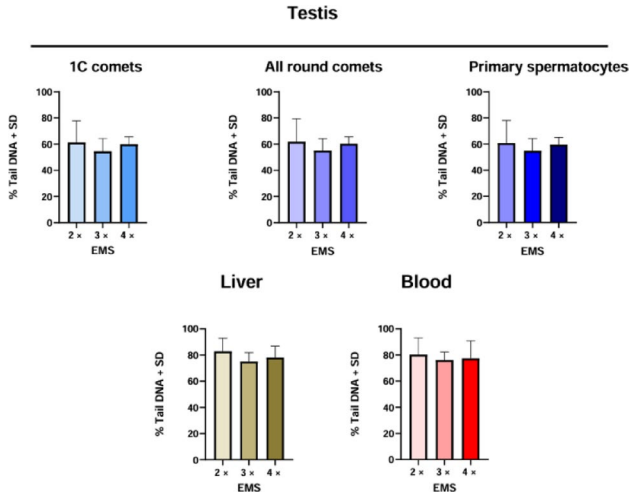

Fig. 5 Comparative analyses of EMS-induced DNA damage in testis, liver and blood after different EMS dose regimens. The x-axes represent the EMS exposure scenarios involving two (2X), three (3X) or four (4X) daily doses administered at 24 h intervals. The y-axes represent % Tail DNA+SD. Statistical differences were determined by comparing the % Tail DNA in each cell exposure scenario for each cell type using ordinary one-way ANOVA with Tukey's post hoc test

Ethyl-methanesulfonate (EMS)

Rats were exposed to 200 mg/kg bw EMS for two, three or four times with 24 h intervals between doses. Body weights were recorded at randomization, before all dosings on all days, and at termination 3 h after the last dosing (Fig. 4, Supplementary Material 2). Following exposure, the normalised body weights at termination significantly decreased in all groups exposed two, three or four times, and with the number of exposures. The absolute left testis and liver weights remained stable (Supplementary Material 2). The relative left testis and liver weights were unaffected after two and three daily exposures, whereas after four daily exposures, both testis and liver relative weights significantly increased.

The DNA damage levels after 2, 3 or 4 doses of EMS 24 h apart are presented as means % Tail DNA+SD in each group (Table 3). DNA damage was induced in the testicular germ cells of both haploid spermatids (1C comets) and PS, in all dose regimens. Moreover, DNA lesions were induced at similar levels in "all round comets" as in 1C and PS, whereas in cells from liver and blood the levels were higher.

Next, we compared the effects of the EMS dose scenarios of two, three or four exposures, 24 h apart, in each cell type. No significant differences were detected within each cell type of the testis or in the somatic cells after the different dose regimens (Fig. 5). Comparing the solvent controls, minor significant changes were observed in 1C, PS and blood cells (Table III; p-values not shown).

Next, we compared DNA damage levels between the different cell types in each dose regimen (Fig. 6**)**. No significant

Table 3 DNA damage levels (% Tail DNA±SD) in the different cell types of the testis, liver and blood of solvent control animals and EMS-treated (200 mg/kg) rats exposed 2, 3, or 4 times, 24 h apart (n=5–6 animals per group)

	2 X solvent control	2 X EMS *p* value[a]	3 X solvent control	3 X EMS *p* value[a]	4 X solvent control	4 X EMS *p* value[a]
1C Comets	0.10±0.04	61.27±16.62 $p<0.0001$	0.18±0.08	54.57±9.78 $p<0.0001$	0.33±0.16	60.80±5.72 $p<0.0001$
"All round comets"	0.23±0.08	61.91±17.54 $p<0.0001$	0.49±0.08	55.14±9.09 $p<0.0001$	0.64±0.19	60.39±5.23 $p<0.0001$
Primary spermatocytes	1.09±0.69	60.83±17.33 $p<0.0001$	2.52±1.35	54.89±9.41 $p<0.0001$	3.30±1.33	59.64±5.58 $p<0.0001$
Blood	0.14±0.07	80.40±12.66 $p<0.0001$	0.19±0.12	76.18±6.12 $p<0.0001$	1.18±0.75	77.48±13.36 $p<0.0001$
Liver	0.08±0.04	82.87±9.99 $p<0.0001$	0.16±0.10	75.03±6.84 $p<0.0001$	0.20±0.13	78.24±8.63 $p<0.0001$

[a]*P* values express statistical significance of the mean % Tail DNA of treated animals compared to control animals using the Student's t-test

differences between the different cell types of the testis were observed in any of the three dose regimens, showing that DNA lesions were induced at similar levels in all testicular cell types, irrespective of exposure scenario. After two doses of EMS 24 h apart, testicular cells showed lower DNA damage than liver and blood; however, the differences were not statistically significant due to inter-animal variability. Following three and four exposures 24 h apart, the variability was lower, and the DNA damage levels in testicular cells were significantly lower than in the somatic tissues. In solvent controls, statistically significant differences were observed, also between testicular cell types, although the absolute differences were minor (data not shown) (Fig. 7).

DNA damage levels in 1C haploid spermatids and > 1C testicular cells

Next, we compared the levels of DNA lesions induced by both X-rays and EMS in 1C haploid spermatids with the other cells of the testicle, the > 1C cells (Fig. 8). No statistical differences in DNA damage levels were observed between 1C and > 1C cells, after exposure to the two genotoxicants. Marginally higher levels in > 1C cells compared to 1C cells were consistently observed for sham (X-rays) or solvent controls (EMS; data not shown).

Variation in control animals

Low variation in % Tail DNA was observed among the control animals (Fig. 8). The lowest variation was observed in

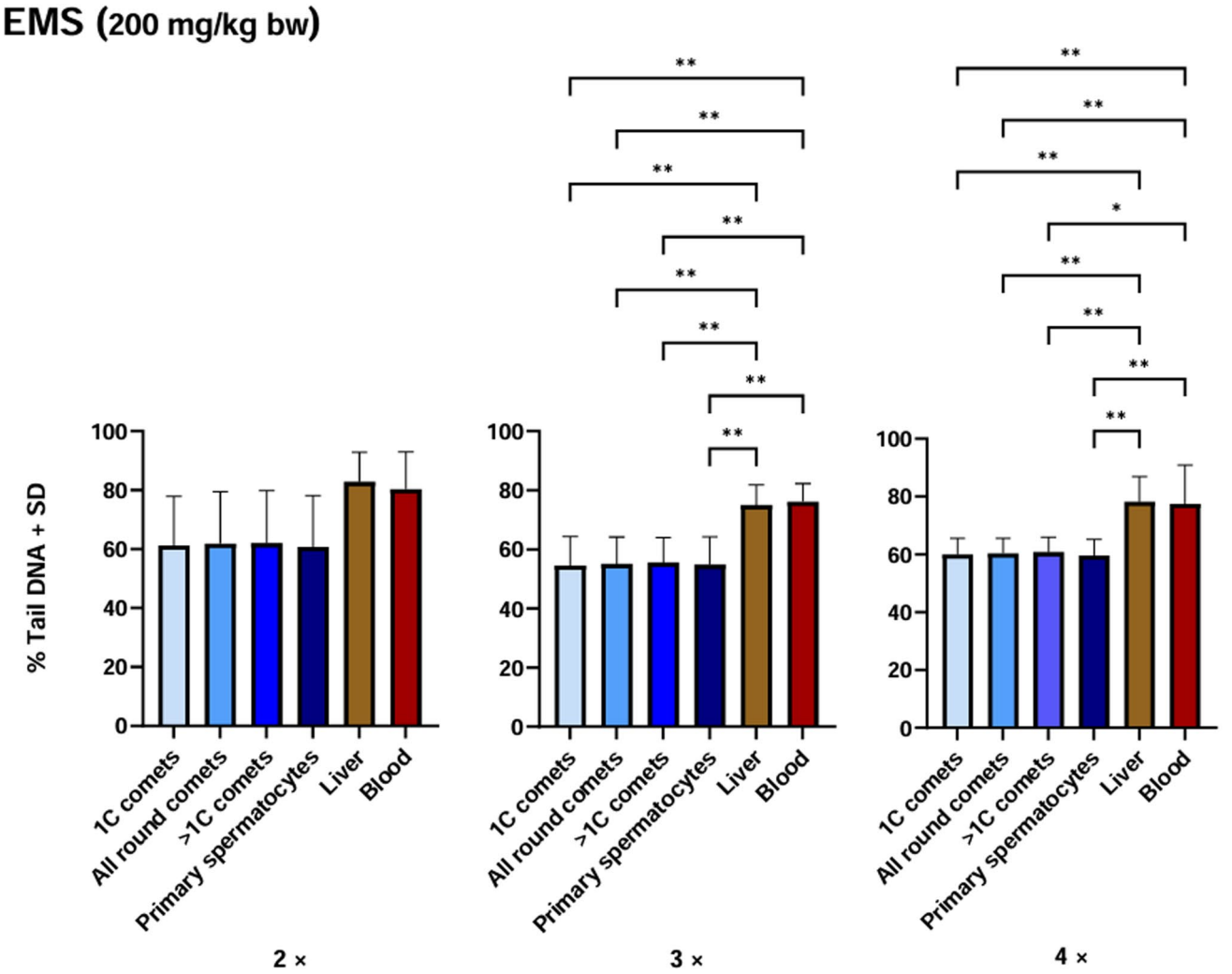

Fig. 6 Comparison of DNA damage levels in testicular and somatic cells after different exposure scenarios with EMS. The x-axes show the different cell types (from left to right 1C comets (light blue), "all round comets" (medium blue), > 1C comets (blue), Primary spermatocytes (dark blue), liver (brown) and blood (red) and the y-axes show the % Tail DNA + SD levels. Statistical differences (*$p <= 0.05$), **$p <= 0.01$), ***$p <= 0.001$) were determined by comparing the % Tail DNA in each cell type using ordinary one-way ANOVA with Tukey–Kramer HSD's post hoc test

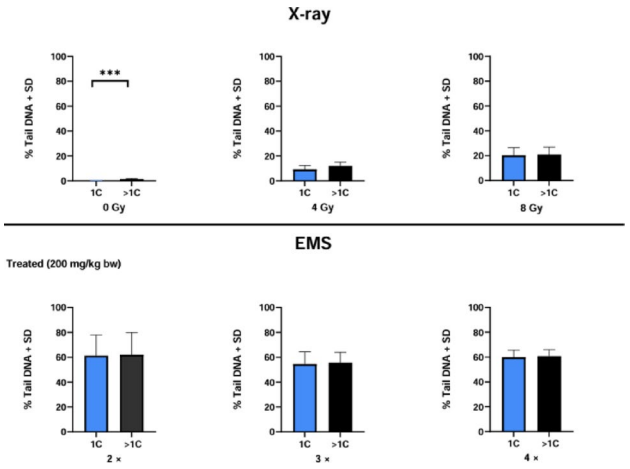

Fig. 7 DNA damage levels in 1C spermatids versus > 1C testicular cells. The upper panel represents results with X-rays and the lower panel with EMS. The x-axes represent the cell type (1C or > 1C) and the y-axes represent the % Tail DNA + SD. For X-rays each graph represents one dose level (0, 4 and 8 Gy). For EMS the graphs represent 2 exposures (2 x, left), 3 exposures (3 x, middle) and 4 exposures (4 x, right) given 24 h apart. Significant differences in % Tail DNA between exposed and solvent control groups are indicated with *($p <= 0{,}05$), **($p <= 0{,}01$), ***($p <= 0{,}001$) using unpaired 2-tailed Student's t-test

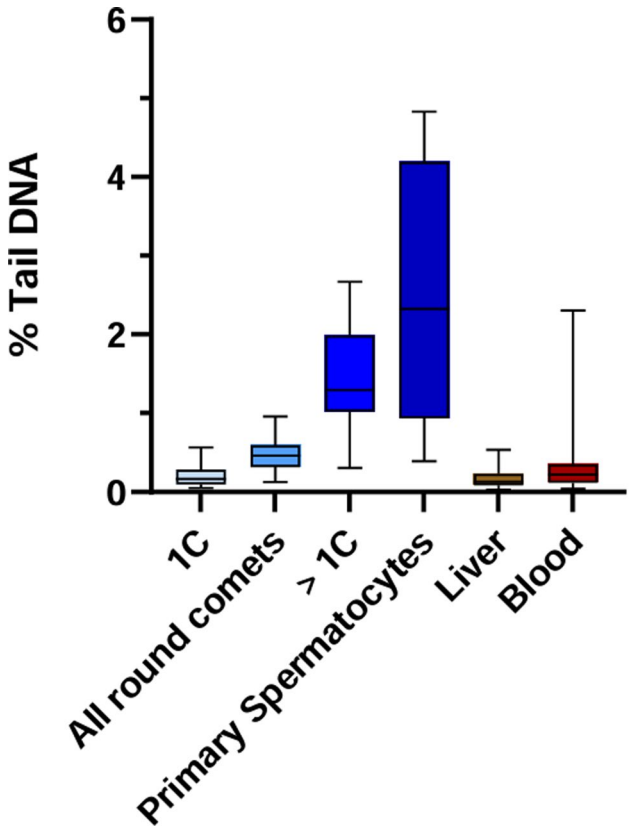

Fig. 8 Variation in control animals in testicular cells, blood and liver. The variation is represented as box and whiskers plots for sham animals in the X-rays experiment (n = 5) and solvent control animals in the EMS experiments (n = 15), (total n = 20). % Tail DNA levels are shown for 1C comets (light blue), "all round comets" (medium blue), > 1C comets (blue), Primary spermatocytes (dark blue), liver (brown) and blood (red). The boxes show the interquartile range from the 25th percentile to the 75th percentile, and the whiskers show the minimum and maximum values. The lines across each box are the median values

the 1C comets and liver comets with interquartile ranges less than 0.2% Tail DNA. The highest variation was observed in PS, however, the interquartile range was only 3.3% Tail DNA. For the exposed groups, the coefficients of variance ranged from 8.0 to 33.8%. The day-to-day variation between the EMS solvent control groups was also low (Table 3); for "all round comets," the values ranged from 0.23 to 0.64 % Tail DNA and for 1C comets the variation was even lower, ranging from 0.1 to 0.3 % Tail DNA.

Power analysis using the Wilcoxon rank-sum test indicated that a sample size of four animals per group was sufficient to detect a two-fold difference between groups with 80% power. This calculation was based on the means and standard deviations from both control (untreated; sham (X-rays) and solvent (EMS) controls) and treated (X-ray and EMS-exposed) groups.

Discussion

The relevance of acquiring germ cell-specific genotoxicity data stems from the fact that the OECD TG 489 does not recommend testing of testicular germ cells because the cell suspension contains a mixture of germ and somatic cells, however, obtaining supportive data directly in the relevant target cell type, i.e., germ cells, aligns with the classification requirements of the GSH (2021) and the CLP Regulation (2008) for Mutagenicity. Hence, the overall project objective is to refine the in vivo comet assay methodology to obtain data on DNA damage induction and removal dynamics specifically in germ cells of the testis, with robust demonstrations of feasibility. Herein, we evaluated our recently developed protocol for obtaining in vivo germ cell-specific genotoxicity data by exposing Wistar rats to the direct-acting genotoxicants X-rays and EMS.

X-ray was selected due to its well-characterized ability to uniformly induce DNA lesions, penetrate tissues, and act independently of metabolic activation or biodistribution. DNA strand breaks in testicular cells and germ cells in male rodents after X-ray exposure have been widely reported using the alkaline comet assay (Cordelli et al. 2003; Dobrzynska 2005, 2007, 2011; Dobrzynska and Radzikowska 2022; Olive et al. 1994; Paris et al. 2011; Villani et al. 2013; Zheng and Olive 1997). EMS was selected as one of the recommended standard positive control compounds in the TG 489 (TG489 2016), with well-characterized direct alkylating action (Gocke et al. 2009). Like X-rays, EMS induces DNA

damage independently of metabolic activation, assuming distribution to the testicle. Moreover, EMS was used in the TG 489. In vivo mammalian alkaline comet assay validation across multiple laboratories to compare results with somatic tissues (liver and stomach) across laboratories (Morita et al. 2015; Uno et al. 2015a, 2015b, 2015c; Uno and Omori 2015), leading to the acceptance by OECD of TG 489.

By studying responses to X-rays and EMS, we assessed the functionality and reliability of the revised in vivo comet assay in testicular germ cells, and explored differences between germ cell types, between 1C and the other of the testis, and with somatic tissues (liver and blood).

Our findings demonstrate that the revised comet assay for testicular germ cells effectively detects DNA damage across testicular cell types (Tables 2 and 3) after exposure to X-rays and EMS. Moreover, valuable supportive data is obtained in the relevant cell type, in germ cells, that aligns with the classification requirements of GHS (2021) and CLP (2008) for the Muta 1B category and reinforces the applicability of extending the current TG 489 guideline to include testicular germ cell assessments.

Variability in comet parameters may represent an underappreciated source of inter- and intra-laboratory inconsistency in comet assay results (Collins et al. 2014). The standardization efforts herein were motivated to address longstanding concerns regarding inter- and intra-laboratory variability in comet assay results (Azqueta et al. 2019; Brunborg et al. 2015; Collins et al. 2014, 2008; Moller et al. 2026), as discussed in the 6th International Workshop on Genotoxicity Testing (IWGT) (Speit et al. 2015) and as recommended for using the comet assay to predict germ cell genotoxicity (Speit et al. 2009). Harmonization was recently also encouraged in two landmark comet reviews that highlight important aspects of the comet assay, including recommendations for describing comet assay procedures and results (Brunborg and Collins 2020; Moller et al. 2020) and consensus protocols (Collins et al. 2023). An analysis of historical negative controls from seven laboratories processed by the 8th IWGT committee highlighted that the variability observed in comet assays can be attributed to both technical factors and biological variation (Dertinger et al. 2023). Similar conclusions, emphasizing the technical factors, have been drawn in previous studies (Azqueta et al. 2011; Brunborg et al. 2018; Collins et al. 2014, 2008). The IWGT report (Dertinger et al. 2023) reinforced these concerns, concluding that standardization of both the animal experiment and the comet assay protocol could substantially reduce data variability.

To limit variability and enhance reproducibility, procedures were thus extensively standardized, including parameters related to the animal experiment, tissue harvest to single-cell preparations, and the comet assay. These included uniformity in physical conditions (e.g. timing between animals/operations, gel electrophoresis conditions), and operations (e.g. exposure, termination, tissue collection, single-cell preparations, embedding in agarose, and subsequent comet assay analyses steps). Rapid animal handling, dissection, and tissue processing, along with maintaining samples on ice at all times, were done to minimize background DNA strand breaks from tissue degradation and nuclease activity, while restricting DNA repair, to limit variability and prevent false positives. DMSO was added to the lysis solution to scavenge potential reactive oxygen species, and electrophoresis conditions were tightly controlled due to their strong impact on % Tail DNA (Brunborg et al. 2018).

Previous studies revealing DNA lesion- and pathway-specific differences in cells of various stages of spermatogenesis of both humans and rodents (Jansen et al. 2001; Olsen et al. 2001, 2003, 2005) indicate that testicular germ cells may respond differently to genotoxic agents than somatic cells, and that rodent models may be poor models for humans. Nucleotide excision repair was inefficient in both human and rodent male germ cells (Jansen et al. 2001; Olsen et al. 2005), oxidized purines (i.e. 8-hydroxyguanine) were poorly repaired in human but efficiently in rodent germ cells (Olsen et al. 2003), whereas DNA lesions induced by methyl methanesulfonate (MMS) were efficiently repaired in both species (Olsen et al. 2001). Since single-strand breaks are known to be rapidly repaired (Caldecott 2008; Janoshazi et al. 2020; Olsen et al. 2001), immediate termination after X-rays or 3 h after EMS was done to limit repair leading to lower and more variable % Tail DNA levels.

The results show low variability (Tables 2 and 3, Fig. 8), with low standard deviations and narrow interquartile ranges across experimental groups, demonstrating the effect of methodological consistency. The solvent control 1C comets % Tail DNA levels were low (range 0.1–0.3 %) with standard deviations of 0.04–0.16. This is illustrated in Fig. 8, showing very low interquartile ranges for 1C comets, "all round comets", >1C comets, blood and liver. In PS, the interquartile range was higher, however, below 3.3 % Tail DNA. In the exposed groups, the coefficients of variances ranged 8.0–33.8 %, which can be considered low. Moreover, day-to-day variations in the EMS solvent controls were low, particularly for 1C comets (0.1–0.3 % Tail DNA). In line with this, power analyses estimated four animals per group reflect the low variation observed, and is lower than an earlier estimate of 7 animals per group to detect similar fold changes in DNA strand breaks in mouse testicular cells (Hansen et al. 2014) and the recommended minimum of 5 animals per group in TG 489 (TG489 2016). The higher blood cell damage after X-rays compared to testicular or liver cells likely reflect repair during the short time between blood harvest and tissue dissection, emphasizing

the importance of post-exposure timing and handling to avoid artifacts (Azqueta et al. 2011; Burlinson et al. 2007; Collins et al. 2008; Koppen et al. 2017; Tice et al. 2000). This is probably most relevant for agents such as X-rays, inducing rapidly repairable lesions and where lesion induction stops immediately after cessation of exposure, whereas chemical genotoxicants may continue to induce lesions due to their persistence in tissues and repair efficiency vary according to the induced type of DNA damage.

The 1C comet population was selected using Tot_Int histograms as exemplified in Fig. 3. The identification of the 1C comet population was confirmed by comparing the Tot_Int levels of "all round comets" with those of the spike-in sample, PS (4C) and somatic (2C–4C) tissues, since the latter cell types do not contain 1C comets (Fig. 3). The Tot_Int threshold–based separation of 1C and > 1C testicular cells may result in some > 1C comets being included in the 1C population when distributions overlap; therefore, conservative threshold settings, as used here, is recommended. Robust distinction and selection of 1C from > 1C comets of "all round comets" of the testicle required scoring of more comets per animal than the standard protocol recommendations of acquiring 150 comets per cell type (TG489 2016). The selected 1C (haploid) comets averaged 46% of "all round comets" (range 11–65%), and thus an average of > 300 round comets should be scored to obtain ~ 150 1C comets, assuming no significant change in distribution of testicular cells. Low "all round comet" numbers scored in five of the animals were due to low comet gel density and volume embedded. Increasing the cell density or volume embedded would resolve this issue (Collins et al. 2023).

For X-ray treatment, total doses of 4 and 8 Gy were chosen based on prior studies in rat testes of up to 10 Gy (10 weeks after irradiation; Abuelhija et al. 2013), and considering the dynamic range of the comet assay. X-rays served as a robust positive control. We found similar strand break levels in germ cells (1C, "all round comets" and PS comets), the testicular somatic cells (> 1C comets and partly enriched PS comets), in somatic cells (liver). Importantly, the immediate termination of animals post-exposure that limited repair, and the standardized protocol facilitated comparable DNA strand break levels. Most in vivo studies with X-rays have used delayed sampling; from 30 min up to months post-exposure (Cordelli et al. 2003; Dobrzynska 2005, 2007, 2011; Dobrzynska and Radzikowska 2022; Olive et al. 1994; Paris et al. 2011). Immediate post-termination processing is crucial for accurate DNA damage assessment after ionizing radiation, as it systematically prevents DNA repair (Caldecott 2008; Janoshazi et al. 2020; Olsen et al. 2001). We previously showed that round spermatids and PS from both humans and rats efficiently repaired single-strand breaks induced by MMS ex vivo (Olsen et al. 2001). To our knowledge, only two in vivo studies have reported immediate termination (Villani et al. 2013; Zheng and Olive 1997). Mice exposed to 5, 10 and 15 Gy of X-rays showed efficient removal in testicular cells ($T_{1/2}$ of 12 min) and in somatic cells ($T_{1/2}$ of 12 (bone marrow) – 45 min), after 15 Gy X-rays (Zheng and Olive 1997). The similar DNA damage levels detected after X-rays (Table 2) confirm that the revised comet assay is suitable to detect similar levels of % Tail DNA following induction of identical DNA damage levels.

EMS was chosen as a direct-acting genotoxic alkylating agent, due to its extensive use in establishing the TG 489 in vivo rodent alkaline comet assay, and its use as a positive control in other OECD TGs for genotoxicity and mutagenicity. The selected dose (200 mg/kg bw) was the same as used in the validation of the TG 489 (Morita et al. 2015; Uno et al. 2015a, 2015b, 2015c; Uno and Omori 2015), with 14 laboratories reporting 11–67 % Tail DNA in liver. The increased relative organ weights for both the testis and liver (Fig. 4), limited to the 4 daily dose scenario, may indicate organ toxicity, or merely reflect the overall body weight reduction since the absolute organ weights did not change significantly. The EMS dose of 200 mg/kg bw was not expected to induce significant testicular toxicity (Morita et al. 2015; Uno et al. 2015a, 2015b, 2015c; Uno and Omori 2015).

Comparative analysis of X-ray and EMS-induced DNA damage between 1C germ cell comets and > 1C testicular comets, representing the other cell types in the testis, including somatic cells, did not reveal notable differences (Fig. 7). To better characterize potential cell-type-specific responses, future studies should evaluate a broader range of chemical exposures.

Clear induction of strand breaks was observed in testicular germ cells, in both 1C, PS and > 1C cells, confirming the applicability of the method for studying germ cell-specific genotoxicity (Tables 2 and 3, Figs. 5 and 6). The data suggest that testicular cells throughout the seminiferous tubules, with variable levels of protection via the testicular gradient barrier, acquire DNA lesions following exposure to X-rays and the small directly alkylating EMS. DNA damage levels in haploid round spermatids and PS exposed to both X-rays and EMS were similar (Fig. 6), which is interesting considering their differences in nuclear organisation (meiotic recombination and chromosome synapsis in PS, preparation for nuclear condensation and acrosome generation in round spermatids), physiology and ploidy. When comparing EMS-induced DNA damage levels in testicular and somatic cells, the levels in testicular cells were generally lower than in somatic cells (Fig. 7), potentially due to factors including the testicular gradient barrier, blood flow and other ADME factors. Animals given two doses of EMS

had higher inter-individual variability than the groups given three or four doses of EMS. There were no significant differences in DNA damage levels within each cell type, neither testicular germ cells nor somatic cells, investigated after two, three or four doses of EMS (Fig. 5). Combining the higher variability in animals given two doses of EMS and the reduced body and relative organ weights after four doses of EMS, three daily doses of EMS seem preferable for follow-up studies. The % tail DNA values in testicular cells (Table 3; 54.6–61.9% Tail DNA) after EMS-exposure are within the comet assay's dynamic range, whereas the higher values observed in blood and liver approached the upper limit (Table 3; 75.0–82.9% Tail DNA). Consequently, differences between somatic cells and testicular cells may be underestimated.

Only a few in vivo studies have reported DNA strand breaks in testicular germ cells following EMS via the oral route using the alkaline comet assay (Anderson et al. 1996; Hansen et al. 2014; Lovaković 2021; O'Donoghue et al. 2021; Sharma et al. 2018). Hansen et al. (Hansen et al. 2014) and Sharma et al. (Sharma et al. 2018) reported clear responses using EMS (300 mg/kg, necropsy after 3 h) given once as a positive control in mixed testicular cells from mice, and O'Donoghue et al. (O'Donoghue et al. 2021) similarly observed clear induction of strand breaks in mixed testicular cells in rats following oral gavage at 150 mg/kg given twice (0 and 21 h, necropsy at 24 h). Anderson et al. (Anderson et al. 1996) exposed rats to EMS (100–300 mg/kg bw) by oral gavage for two weeks, with termination two weeks later, and observed no effects in haploid testicular cells or dose–response relationships; this was likely due to delayed sampling and cell preparation at 32 °C, both facilitating DNA repair. Genotoxicity of MMS has also been investigated in vivo using the alkaline comet assay (Crebelli et al. 2019; El Ramy et al. 2007; Manjanatha et al. 2011), with none separating the germ cells, and all reported clear induction of DNA lesions in mixed testicular cell populations.

Our approach provides relevant data for the germ cells of the testicle that may serve as supportive evidence to meet the requirements for Category 1B mutagenicity classification (CLP 2008; GHS 2021). Moreover, it provides insights regarding potential differences in susceptibility between germ and somatic testicular cells. The 1C germ cells reside near the tubule lumen and may experience lower circulating genotoxicant levels, and aggregate testis comet data could potentially misrepresent germ-cell burden. However, addressing all cells in the testicle (as is done in OECD TG 488 Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays (TG488 2025)) may be more pragmatic and conservative, as it may reduce the likelihood of underestimating the potential genotoxic hazards to testicular germ cells.

Using the direct-acting genotoxins X-rays and EMS, our standardized revised comet method demonstrated that total comet intensity can distinguish between 1C and > 1C testicular cells, enabling successful retrieval of germ cell–specific data. Both germ cells and somatic cells showed comparable DNA damage levels following exposure to X-rays and EMS across all testicular cell types examined. Our findings support the applicability and robustness of the revised comet assay protocol for assessing DNA damage in testicular germ cells at different stages of spermatogenesis, highlighting its potential for both regulatory implementation and basic research applications. Beyond regulatory use, the assay may facilitate studies of DNA damage dynamics in germ cells, including the temporal induction and repair of DNA lesions, the capacity of germ cells to process DNA damage, and differential responses across stages of spermatogenesis. Further studies are warranted to explore dose–response relationships, assay specificity (including responses to non-genotoxic compounds and non-genotoxic reprotoxicants), and potential differences among germ cell populations. Additional evaluation of frozen testis tissue or cells could increase assay flexibility and enable analyses of archived samples, while integration of enzymatic modifications (e.g., Fpg treatment) may provide mechanistic insight and improve assay sensitivity. Collectively, these efforts will help refine the methodology and support more comprehensive genotoxicity assessments of both germ and somatic cells in alignment with global regulatory requirements for hazard identification and classification.

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Author contributions The study conception and design were performed by Ann-Karin Hardie Olsen, Yvette Dirven, Gunnar Brunborg and Anoop Kumar Sharma. Animal experiments and material preparation were performed by Ann-Karin Hardie Olsen, Xiaoxiong Ma, Yvette Dirven and Anoop Kumar Sharma. Data collection and analysis were performed by Congying Zheng, Xiaoxiong Ma, Ann-Karin Hardie Olsen and Anoop Kumar Sharma. The templates for selection of comets were generated by Dag Marcus Eide and Xiaoxiong Ma. The first draft of the manuscript was written by Ann-Karin Hardie Olsen and Anoop Kumar Sharma, and all authors approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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