Published in final edited form as:

Mutat Res Genet Toxicol Environ Mutagen. 2015 July ; 0: 172–181. doi:10.1016/j.mrgentox.2015.03.003.

Comet assay evaluation of six chemicals of known genotoxic potential in rats

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Abstract

As a part of an International validation of the *in vivo* rat alkaline comet assay (comet assay) initiated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) we examined six chemicals for potential to induce DNA damage: 2-acetylaminofluorene (2-AAF), *N*-nitrosodimethylamine (DMN), *o*-anisidine, 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), sodium chloride, and sodium arsenite. DNA damage was evaluated in the liver and stomach of 7- to 9-week-old male Sprague Dawley rats. Of the five genotoxic carcinogens tested in our laboratory, DMN and 1,2-DMH were positive in the liver and negative in the stomach, 2-AAF and *o*-anisidine produced an equivocal result in liver and negative results in stomach, and sodium arsenite was negative in both liver and stomach. 1,2-DMH and DMN induced dose-related increases in hedgehogs in the same tissue (liver) that exhibited increased DNA migration. However, no cytotoxicity was indicated by the neutral diffusion assay (assessment of highly fragmented DNA) or histopathology in response to treatment with any of the tested chemicals. Therefore, the increased DNA damage resulting from exposure to DMN and 1,2-DMH was considered to represent a genotoxic response. Sodium chloride, a non-genotoxic non-carcinogen, was negative in both tissues as would be predicted. Although only two (1,2-DMH and DMN) out of five genotoxic carcinogens produced clearly positive results in the comet assay, the results obtained for *o*-anisidine and sodium arsenite in liver and stomach cells are consistent with the known mode of genotoxicity and tissue specificity exhibited by these carcinogens. In contrast,

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Conflict of interest

C. Hobbs, L. Recio, M. Streicker, and M. Boyle are employees of Integrated Laboratory Systems, Inc., a contract research organization offering the comet assay and other services.

given the known genotoxic mode-of-action and target organ carcinogenicity of 2-AAF, it is unclear why this chemical failed to convincingly increase DNA migration in the liver. Thus, the results of the comet assay validation studies conducted in our laboratory were considered appropriate for five out of the six test chemicals.

Keywords

In vivo comet assay; 2-Acetylaminofluorene; *N*-Nitrosodimethylamine; *o*-Anisidine; 1,2-Dimethylhydrazine dihydrochloride; Sodium arsenite

1. Introduction

The *in vivo* rodent alkaline comet assay (comet assay) is used worldwide for detecting DNA damage induced by chemical exposure. The comet assay is increasingly accepted by regulatory agencies for use in evaluating the genotoxic potential of chemicals. It is currently expected to become a second standard *in vivo* genotoxicity assay recommended in the ICH-S2(R1) guidance [1] to supplement data obtained using the *in vivo* micronucleus assay in bone marrow and/or peripheral blood. The comet assay testing protocols have been discussed in detail during meetings of the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published [2–4]. However, the assay has not been formally validated using a standardized study protocol in multiple laboratories. Therefore, the Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an International validation study of the *in vivo* comet assay, in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Center for the Validation of Alternative Methods (ECVAM), and the Mammalian Mutagenicity Study Group (MMS)/Japanese Environmental Mutagen Society (JEMS). The purpose of this International validation study was to refine the *in vivo* comet assay protocol, evaluate the interlaboratory reproducibility of the assay, and evaluate the ability of the assay to identify and differentiate between genotoxic and non-genotoxic chemicals. The results of this validation study were submitted to the Organization for Economic Co-operation and Development (OECD) for use in the establishment of an OECD test guideline for the Comet assay.

Our laboratory participated in the 2nd step of the 4th phase of this International validation study by examining six coded chemicals, assigned by JaCVAM, in the comet assay in male rats. The chemicals included one non- genotoxic chemical, sodium chloride, and five known genotoxic carcinogens: 2-acetylaminofluorene (2-AAF), *o*-anisidine, *N*-nitrosodimethylamine (also known as dimethylnitrosamine; DMN), 1,2-dimethylhydrazine dihydrochlo-ride (1,2-DMH), and sodium arsenite. The five genotoxic carcinogens reflect different mechanisms of DNA damaging activity, including alkylation (DMN, 1,2-DMH), adduct formation (2-AAF, *o*-anisidine), and possible methylation and/or crosslinking (sodium arsenite). Four of the chemicals are known to require metabolic activation for genotoxic activity (DMN, 1,2-DMH, 2-AAF, *o*-anisidine). The studies were conducted according to the validation protocol standardized by JaCVAM [5]. Hedgehogs were

tabulated and histopathological examination of tissues was conducted to obtain measures of cytotoxicity to aid in the interpretation of the comet assay data. As an additional measure of cytotoxicity, we assessed for the presence of cells containing small fragments of DNA, which is potentially indicative of apoptosis or necrosis [2,4].

2. Materials and methods

The studies were conducted in accordance with the validation study protocol (version 14.2) [5]. The actual testing conditions of the studies are described in the following sections. Reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless indicated otherwise; NaOH was purchased from Sigma–Aldrich and from Fisher Scientific (Pittsburgh, PA, USA).

2.1. Test chemicals

Coded samples of 2-AAF (53-96-3), DMN (62-75-9), *o*-anisidine (90-04-0), 1,2-DMH (306-37-6), sodium chloride (7647-14-5), and sodium arsenite (7784-46-5) were provided by JaCVAM for testing in the *in vivo* comet assay. Ethyl methanesulfonate (EMS; 62-50-0), used as a concurrent positive control chemical in each study, was purchased from Sigma–Aldrich Co., (St. Louis, MO, USA) and WAKO Biochemicals (Osaka, Japan). Corn oil and normal saline were used as vehicles and were purchased from Sigma–Aldrich and Ricca Chemical Company (Arlington, TX, USA), respectively.

2.2. Animals

Male Sprague Dawley rats (Charles River Laboratories, Durham, NC, USA) were maintained in a temperature and humidity controlled animal room with a 12-h light/12-h dark cycle within an AAALAC-accredited specific pathogen free facility. Animals were singly housed in polycarbonate cages with absorbent hardwood bedding (Northeastern Products Corp., Warrensburg, NY, USA) and environmental enrichment. Certified rodent chow no. 5002 (Ralston Purina Co., St. Louis, MO, USA) and tap water were provided ad libitum. The studies were approved by the ILS Institutional Animal Care and Use Committee and all procedures were conducted in compliance with the Animal Welfare Act Regulations, 9CFR 1-4, and the Guide for the Care and Use of Laboratory Animals [6].

2.3. Animal treatment

Rats were 7 to 9 weeks of age at the time of chemical treatment. Doses were based on the results of dose setting studies; the highest dose selected was that which produced signs of toxicity such that higher dose levels would have been expected to result in severe animal distress or lethality. In some cases, doses were selected by the validation management team (VMT) on the basis of equivocal results or histopathology findings in validation studies conducted in other laboratories. For each study, following one week of acclimation, 5 rats per treatment group (randomized using a body weight stratification scheme) were administered test chemical or vehicle by oral gavage once daily on day 1, day 2 (24 h later), and day 3 (21 h later). For all studies, the positive control chemical, EMS, was administered twice at 200 mg/kg/day in saline by oral gavage at an interval of 21 h beginning on day 2.

All dosing solutions were prepared fresh daily and administered (at 10 ml/kg body weight) within 2 h of formulation.

2.4. Tissue preparation

Three hours after the final dose, animals were humanely euthanized by exsanguination after being anesthetized using CO_2. The stomach and liver were removed, rinsed with cold mincing buffer [Mg^{+2}, Ca^{+2}, and phenol free Hank's balanced salt solution (Life Technologies, Carlsbad, CA, USA) with 20 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4–7.7 and 10% v/v fresh dimethyl sulfoxide (DMSO)] sufficiently to remove residual blood, and held on ice briefly until processed. A portion of the left lobe of the liver was placed in a vial containing cold mincing solution and rapidly minced until finely dispersed. The glandular portion of the stomach was flushed with mincing solution and incubated on ice in cold mincing solution for 15 min. After incubation, the surface epithelium was gently scraped two times using the back of a scalpel blade; this layer was discarded and the gastric mucosa rinsed with cold mincing buffer. Scraping was repeated 4–5 more times to release cells into 0.5 ml of mincing buffer which was transferred to a microfuge tube. Single cell preparations were maintained on ice until further processing. Additional sections obtained from the same liver lobe and a small portion of the glandular stomach were fixed in 10% neutral buffered formalin (NBF) (Leica Biosystems, Richmond, IL, USA) for 24 h for subsequent histopathology evaluation.

2.5. Comet assay

Comet slides were prepared within one hour following animal sacrifice. Just prior to use, each cell suspension was shaken gently to mix the cells and placed back on ice for 15–30 s to allow clumps to settle. A portion of the supernatant was empirically diluted with a fresh aliquot of 0.5% NuSieve GTG low melting point agarose (Lonza, Rockland, ME, USA) dissolved in Dulbecco's phosphate buffer (Ca^{+2}, Mg^{+2}, and phenol free) at 37 °C, and layered onto microscope slides pre-coated with 1% normal melting agarose. Low-melting point agarose (0.5% (w/v)) was applied as a top layer over the gel-embedded cells. Multiple slides per sample were prepared in a laboratory with a relative humidity of ≤60%. After incubating at least 1 h in cold lysing solution [25 M NaCl, 100 mM Na_2EDTA, 10 mM tris(hydroxymethyl) aminomethane (tris), pH 10, with freshly added 10% DMSO and 1% Triton X-100], one slide per sample (for the neutral diffusion assay) was rinsed with neutralization solution (0.4 M Trizma base, pH 7.5) to remove remaining detergents. The remaining slides were immersed in chilled lysing solution overnight in a refrigerator protected from light. The following day, the slides were rinsed in neutralization solution, randomly positioned in a submarine-type electrophoresis unit and treated with cold alkali solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min to allow DNA unwinding, then electrophoresed at 1–9 °C for 20 min at 25 V (0.7 V/cm), with a current of approximately 300 mA. Following electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for 5 min and then dehydrated by immersion in ice-cold 100% ethanol (Pharmco-AAPER, Shelbyville, KY, USA) for ≥5 min. Air-dried slides were stored at room temperature in a desiccator with a relative humidity of <60%.

After staining slides with SYBR® Gold (Molecular Probes, Invitrogen, Carlsbad, CA, USA), 100 cells were scored per sample using comet IV image analysis software (Perceptive Instruments, Ltd., Suffolk, UK). Slides were coded and scored without knowledge of their identity. Cells were classified into three categories: scorable, non-scorable, and "hedgehog". The following cases of analyzable cells were excluded from the quantitative analysis: (a) the recognition by the software was considered to be incorrect; (b) the overall staining of the nucleus and/or migrated DNA was considered poor; and (c) contained ≥90% DNA in the tail. For each scorable cell, the extent of DNA migration was characterized using the % tail DNA measurement; however, tail length and Olive tail moment (OTM) measurements were also collected. Cells defined as "hedgehogs" by the criteria outlined in the validation protocol were tabulated as they were encountered in the process of scoring 100 cells. Because unscorable non-hedgehog cells were not tabulated during this process, the hedgehog measurement represents a qualitative assessment of hedgehogs within each cell preparation, rather than a percentage, and no statistical analyses were conducted on the hedgehog endpoint.

2.6. Neutral diffusion assay

Under neutral conditions in the absence of an applied electrical field, cells with extensive DNA degradation, potentially indicative of apoptosis or necrosis [2,4,7], exhibit a highly diffuse pattern of DNA compared to the condensed pattern associated with intact strands of high molecular weight DNA. To measure the incidence of cells with low molecular weight (LMW) DNA, after incubation for 1 h in lysis buffer a slide for each sample was neutralized with 0.4 M Trizma base (pH 7.5), fixed in 100% ethanol, air-dried, and stored in a desiccator at room temperature. After staining slides with SYBR Gold®, slides were scanned and 100 cells per slide were categorized as having either condensed DNA (type I) or diffused LMW DNA (type II).

2.7. Histopathology

Liver and stomach tissues from animals from all but the 2-AAF study were evaluated by histopathology (no tissues were collected for histopathology in the 2-AAF study). Sections of liver and stomach were fixed in 10% NBF, processed, and embedded in paraffin. Tissues were sectioned at 5 mm thickness and stained with hematoxylin and eosin (H&E) for microscopic evaluation. With the exception of one case in which stomach tissues for all dose groups were analyzed, only tissues from vehicle control and high dose animals were evaluated.

2.8. Statistics

The comet and neutral diffusion assay data were analyzed using statistical analysis system software, version 9.2 (SAS Institute, Cary, NC, USA). For comet data, the medians of the 50 cells scored on each of two slides for each animal were averaged and the resulting individual animal means were used to calculate dose group means. For LMW and hedgehog data, the mean of the total cells scored for each animal was used to calculate dose group means. A two-tailed Dunnett's test ($p < 0.05$) was used to compare each dose level to the concurrent control and a linear trend test ($p < 0.05$) was used to determine the presence of a dose

response. Criteria for a positive test were the presence of at least one positive dose group and a positive trend test. A test was judged to be equivocal if only a significant trend or a single significant dose group was observed. Negative tests were those in which neither a significant trend nor a significant dose group were seen. Laboratory historical control data were not considered in the final determination of a positive or negative result. A one-tailed Student's *t*-test ($p < 0.025$) was used to verify a positive response to the control compound, EMS.

3. Results

3.1. 2-AAF

2-AAF was administered to Sprague Dawley rats at 0, 250, 500, and 1000 mg/kg/day in corn oil. The decision to set the top dose level at 1000 mg/kg in the comet assay was based on adverse clinical observations, weight loss, and some suppression of reticulocytes measured in the bone marrow in dose setting studies. Over the 3-day course of the validation study, a 10% reduction in body weight was noted in the 1000 mg/kg dose group; a 5.5% and 5.7% reduction was measured in the 250 and 500 mg/kg dose groups, respectively (data not shown). One animal in the low dose group was observed to have transient diarrhea and another in the middle dose group exhibited alopecia of a foreleg. Two animals in the top dose group exhibited decreased movement on day 2 of the study. Orange-stained bedding was observed in the cages of top dose group animals during the last two days of the study. All other animals appeared normal throughout the course of the study.

Under the conditions used in the comet assay, a statistically significant ($p = 0.0233$) increase in the % tail DNA was measured only in the liver of rats administered the lowest concentration of 2-AAF (250 mg/kg); no increase in damage was evident at higher doses or in the stomach (Table 1). The concurrent positive control chemical, EMS, produced significant increases in DNA damage in both the liver and the stomach. Although histopathological evaluation was not conducted on the tissues collected in this study, a neutral diffusion assay was conducted in parallel with the comet assay to assess chemical-induced cytotoxicity in the form of LMW DNA, thought to be indicative of necrosis or early stages of apoptosis [2,4,7]. A summary of the neutral diffusion assay results is provided in Table 7. No dose-related increases in the percentage of cells containing LMW DNA were observed in the livers of animals treated with 2-AAF. However, a statistically significant ($p = 0.0123$) increase in LMW DNA cells was measured in the stomach of animals treated with the lowest dose (250 mg/kg) of 2-AAF. There was no increase in the relative number of hedgehogs in liver or stomach of 2-AAF-treated animals (Table 1). In animals administered EMS, no increase was discerned in the percentage of cells containing LMW DNA; an increased number of hedgehogs was seen in the stomach but not the liver (Table 1). On the basis of these results and the criteria set forth in the validation study protocol, 2-AAF was equivocal in the liver and negative in the stomach in the comet assay.

3.2. DMN

The dose levels of DMN were 0, 0.63, 1.25, and 2.5 mg/kg/day. These doses and the vehicle (normal saline) were selected by the VMT based on results of a previous study conducted in

another laboratory. In the earlier study, positive results seen over a dose range of 2.5–10 mg/kg/day were confounded by excessive toxicity as determined by histopathological evaluation. In our validation study, a 6.3% increase in body weight from day 1 to day 3 was measured in the highest dose group as compared to a 4.5% increase in the vehicle control group (data not shown). All study animals appeared normal throughout the entire course of the study.

Over the range of doses tested in the comet assay, no significant increases in the % tail DNA were observed in stomach tissue of rats administered DMN (Table 2). Cytotoxicity was not indicated in the stomach of treated animals as assessed by the percentage of cells containing LMW DNA (Table 7), or by histopathological evaluation of H&E-stained tissue sections. In liver, there was a significant ($p < 0.0001$) increase in % tail DNA for all three doses of DMN tested, and a positive trend test ($p < 0.0001$; Table 2). An increase in the number of hedgehogs was observed in liver tissue at the middle and top dose; no cytotoxicity in liver was indicated by the neutral diffusion assay (Table 7). Histopathological evaluation did not reveal findings considered to be related to chemical treatment. In the EMS-treated rats, a statistically significant increase in % tail DNA was measured in the liver and stomach (Table 2); the relative number of hedgehogs was increased in stomach but not liver. Under the conditions used in the validation study, DMN was positive in liver but negative in stomach.

3.3. o-Anisidine

The dose levels of *o*-anisidine selected for the comet assay were 0, 150, 300, and 600 mg/kg/day, administered in corn oil. Dose selection was based on the adverse clinical observations at doses >600 mg/kg in a dose setting study. In the validation study, a 3.1% loss in body weight from day 1 to day 3 was measured in the 600 mg/kg group as compared to a 6.9% increase in the vehicle control group (data not shown). Some animals in each of the *o*-anisidine treatment groups exhibited an ungroomed appearance, lethargy, hunched posture, and/or decreased movement on day 1 of dosing; the majority of animals returned to normal throughout the remainder of the study. However, one animal in the 300 mg/kg dose group and one animal in the 600 mg/kg group exhibited red ocular discharge on day 2. One animal administered EMS was observed to have a rough coat on day 2 of the study. All other animals were observed to be normal throughout the course of the study.

A statistically significant ($p = 0.0182$) increase in % tail DNA was detected in the liver of animals administered 600 mg/kg *o*-anisidine without a corresponding significant dose response; no induction of DNA damage was evident in the stomach (Table 3). No significant increases in hedgehogs were observed in liver or stomach tissues in animals administered *o*-anisidine (Table 3). A statistically significant increase in % tail DNA was measured in the liver and stomach of animals administered the positive control chemical, EMS. The occurrence of hedgehogs was increased in the stomach but not in the liver of EMS-treated rats; evidence of cytotoxicity in the stomach was provided by the neutral diffusion assay ($p = 0.0034$). No dose-related increases in the percentage of cells containing LMW DNA were measured in the liver or stomach of animals treated with *o*-anisidine (Table 7). Histopathological evaluation of tissue sections from vehicle control and high dose animals

revealed a test article-related decrease in the amount of glycogen (1/5) and increase in mitosis (2/5) in the hepatocytes. No significant stomach lesions were associated with exposure of rats to *o*-anisidine in this study. Under the conditions used in this validation study, *o*-anisidine was negative in the comet assay in both liver and stomach.

3.4. 1,2-DMH

The dose levels of 1,2-DMH selected for the comet assay were 0, 1.56, 3.13, and 6.25 mg/kg/day, administered in normal saline. The doses and the vehicle were selected by the VMT based on the outcome of a previous study conducted at another laboratory. In the earlier study, positive results seen over a dose range of 6.25–25 mg/kg/day were confounded by excessive toxicity, as revealed by histopathological evaluation. In our study, a 1.6% increase in body weight from day 1 to day 3 was measured in the highest dose group as compared to a 4.5% increase in the untreated control group (data not shown). All study animals appeared normal throughout the entire course of the study.

In the liver, there was a significant ($p < 0.0001$) increase in % tail DNA for all three doses of 1,2-DMH and a significant trend ($p < 0.0001$; Table 4). A dose-related increase in the number of hedgehogs was also observed in the liver. No increases in % tail DNA or hedgehogs were observed in the stomach tissue of treated rats. A statistically significant increase in % tail DNA was measured in the liver and stomach of animals administered the positive control chemical, EMS. Hedgehogs were increased in the stomach but not in the liver of EMS-treated rats.

Under the conditions of the assay, no chemical-related increases in the percentage of cells containing LMW DNA were measured in the liver or stomach of animals treated with either 1,2-DMH or EMS (Table 7). Histopathologic evaluation of the liver and stomach from vehicle control and high dose group animals revealed no significant stomach or liver lesions associated with exposure of rats to 1,2-DMH in this study. Under the conditions used in the validation study, 1,2-DMH was positive in the comet assay in liver tissue but negative in stomach.

3.5. Sodium chloride

On the basis of solubility information provided by the VMT and discussion between the chemical manager and the VMT, deionized water was selected as the most suitable vehicle for sodium chloride. Based on the absence of adverse clinical observations, weight loss, or suppression of reticulocytes in the blood and bone marrow in a dose setting study, the dose levels of sodium chloride selected for the comet assay were 0, 500, 1000, and 2000 mg/kg/day. All animals receiving sodium chloride appeared normal throughout the entire course of the validation study. A 5.3% increase in body weight from day 1 to day 3 was measured in the highest dose group, compared to an 8.2% increase in the untreated control group (data not shown). One animal administered EMS exhibited a hunched posture and another exhibited a rough coat 1 h following dosing. These animals were observed to be normal at the 3-h time point just prior to necropsy.

No increases in % tail DNA or hedgehogs were observed in liver tissue in response to administration of sodium chloride (Table 5). A statistically significant increase in % tail DNA was measured in both the liver and stomach of animals administered EMS. The number of hedgehogs was increased in the stomach, but not in the liver, of EMS-treated rats. No dose-related increase in the percentage of cells containing LMW DNA was measured in the liver or stomach of animals treated with either sodium chloride or EMS (Table 7).

Liver sections from vehicle control and high dose animals and stomach sections from control, low, mid, and high dose group animals were submitted for histopathologic evaluation. Minimal to mild acute inflammation was observed in a dose-dependent manner in the stomach of rats exposed to sodium chloride; evidence of moderate chronic active inflammation and mild acute inflammation was observed in the stomach of one animal in the 2000 mg/kg/day dose group. No treatment-related findings were observed in the liver. Under the conditions used in the assay, sodium chloride was negative in the comet assay in both liver and stomach.

3.6. Sodium arsenite

Sodium arsenite was tested in the comet assay at 7.5, 15, and 30 mg/kg/day, administered in normal saline. These doses were selected by the VMT on the basis of equivocal results obtained in a previous study conducted at another laboratory using these same doses in saline. In our study, no significant increases in % tail DNA were observed in liver or stomach tissue of rats administered sodium arsenite.

Over the concentration range of sodium arsenite tested, no cytotoxicity was observed in the liver or stomach, as assessed by measuring either the presence of hedgehogs or the percentage of cells containing LMW DNA (Table 7). Histopathologic evaluation of the livers from the high dose animals revealed exposure-related nuclear enlargement in hepatocytes (5/5) and bile duct epithelium (3/5) along with a decreased amount of glycogen (5/5) and single cell necrosis in the hepatocytes (1/5), compared with the vehicle control animals. No test article-related effects were observed in the glandular stomach.

The positive control chemical, EMS, induced a significant increase in DNA damage in both the liver and stomach, with a corresponding increase in hedgehogs (Table 6), but not LMW DNA (Table 7). On the basis of these results and the criteria set forth in the validation study protocol, sodium arsenite was judged to give equivocal results in the comet assay in the liver and negative results in the stomach.

4. Discussion

Five of the coded chemicals we tested in the comet assay validation study are known genotoxic carcinogens. Of these, two tested positive (DMN; 1,2-DMH), two tested equivocal (2-AAF, *o*-anisidine), and one tested negative (sodium arsenite) in our studies. The non-genotoxic non-carcinogenic compound, sodium chloride, tested negative in liver and stomach.

4.1. DMN

The metabolic activation of DMN by CYP2E1 and alkylation of DNA are integral to its carcinogenic effect, leading to induction of liver tumors in male rats treated by subcutaneous injection and tumors of nasal cavities and kidneys in rats following inhalation exposures [8,9]. DMN was previously shown to be positive in bacterial mutagenicity and *in vitro* chromosomal aberration tests with metabolic activation [10–12], and in *in vivo* genotoxicity tests, including the unscheduled DNA synthesis (UDS), micronucleus (MN), and transgenic rodent (TGR) mutation assays [13–17]. DMN also tested positive in various tissues, including liver of rats, and liver and stomach of mice, in previous comet studies [18–20], and consistent with those results, it also tested positive in liver of rats in our validation study. The number of hedgehogs was increased in a dose-related fashion in the liver of treated rats, but no lesions suggestive of cytotoxicity were revealed by histopathological analysis of either liver or stomach tissues, and no increases in LMW DNA were observed in either tissue.

4.2. 1,2-DMH

1,2-DMH produces adenomas and adenocarcinomas in the colon of mice and rats [21]. It is metabolized by a sequence of oxidation steps; the last metabolite decomposes to give the highly reactive methyldiazonium ion (DNA alkylation) to which the carcinogenicity of the compound has been attributed [22]. In previous genotoxicity testing, 1,2-DMH was positive in bacterial mutagenicity and *in vitro* chromosome aberration tests, as well as in UDS, MN, and comet assays in rodents [10,13,14,18,19,23–25]. In the previous comet studies, 1,2-DMH was reported to induce DNA damage in a variety of rat tissues, including liver [18,25] and stomach [25]. In our study, designed on the basis of histopathological toxicity information gathered from an earlier validation study conducted in another laboratory, 1,2-DMH tested positive in liver at all doses, while no increases in DNA damage were seen in stomach tissue up to the highest dose of 6.25 mg/kg/day. The positive result in rat stomach reported in a previously published comet study [25] might have resulted from the single administration of a much higher concentration of test article (100 mg/kg). A strong dose-related increase in the number of hedgehogs was seen in liver tissues of 1,2-DMH-treated rats, but histopathological examination of liver and stomach tissues revealed no evidence of necrosis or apoptosis in either tissue. In addition, no increases in LMW DNA were seen in liver or stomach tissue of 1,2-DMH-treated rats.

4.3. Sodium arsenite

Arsenic is considered a human carcinogen primarily on the basis of epidemiological studies. There is limited experimental evidence for its carcinogenicity in animals. A small number of benign and malignant tumors including lung adenomas and carcinomas, kidney adenomas/papillomas and carcinomas, and urinary bladder carcinomas were observed in female rats following administration of sodium arsenite in drinking water; tumor incidence lacked statistical significance in this 2-year study [26]. Transplacental exposure during gestation induced lung, liver, ovary, uterus, and adrenal tumors in the offspring of mice in several studies [27,28]. Arsenicals are not DNA-reactive, but reportedly can induce DNA-protein crosslinking [29]. Low concentrations of trivalent arsenicals produce oxidative DNA

damage and interfere with spindle function [29,30]. Observation of altered DNA methylation and aberrant gene expression following transplacental exposure to arsenic at a hepatocarcinogenic dose implies a link between DNA methylation and arsenic carcinogenesis [31]. Furthermore, a role for the metabolic methylation of arsenic in the liver is suggested by a study in which maintenance of mice on a choline-deficient diet was associated with a decrease in hepatic methyl donor pool availability, reduced susceptibility to bone marrow genotoxicity, and a shift of tissue specificity for arsenic-induced DNA damage from liver and bladder to skin following acute treatment [32]. In previous genotoxicity testing, sodium arsenite was not mutagenic to bacteria but was clastogenic in a mammalian chromosome aberration assay, and induced micronuclei in the bone marrow of mice [10,11,32,33]. In previous comet assays, sodium arsenite induced DNA damage in bone marrow and testicular cells of mice and in blood, liver, and kidney of rats following long-term exposure [34,35]. In contrast, relatively high acute oral exposure to sodium arsenite resulted in decreased DNA migration, indicative of DNA crosslinking [32]. Based on the available mechanistic information, the VMT concluded that sodium arsenite would be expected to be negative for induction of DNA damage detectable by the comet assay in stomach and liver [5]. Consistent with this prediction, administration of sodium arsenite to Sprague Dawley rats did not result in increased % tail DNA in the liver or stomach in our validation study.

4.4. 2-AAF

2-AAF is a well-known genotoxic carcinogen, inducing tumors in many organs, including liver, urinary bladder, and kidney, in many animal species [36]. Metabolic activation of 2-AAF is required for its genotoxic and carcinogenic activity; the first step is the conversion of 2-AAF to the *N*-hydroxy derivative capable of forming DNA bulky adducts [37]. In previous genetic toxicity evaluations, 2-AAF tested positive in bacterial mutagenicity and *in vitro* chromosome aberration tests and in a variety of *in vivo* tests including the UDS, MN, and Big Blue® mutation assays in rats and/or mice [11,13,14,17,23,38]. It was also reported to be positive in the comet assay in colon, liver, kidney, and lung cells of mice administered 600 mg/kg 2-AAF [19,25]. In our validation study, 2-AAF was negative in stomach and equivocal in liver (based on a statistically significant increase in % tail DNA in the lowest dose group). Adverse clinical symptoms and suppression of bone marrow reticulocytes observed in dose setting studies indicate that the top dose of 1000 mg/kg/day in the validation study met the criteria for a valid test. The equivocal result in our study is consistent with the overall judgment of the VMT in the 1st step of the 4th phase validation study, in which 2-AAF tested negative in two of three laboratories; the third laboratory reported a positive result in the liver [5]. In another report, 2-AAF failed to increase DNA damage in rat liver, as measured by the comet assay, when administered orally; whereas, increased DNA damage was detected in the liver following intraperitoneal injection [39,40]. This suggests that the route of administration and dose achieved may be important factors in the ability to detect 2-AAF-induced genetic damage. Nevertheless, given its genotoxic mode-of-action, it is unclear why 2-AAF failed to convincingly increase DNA migration in the liver in this and previous validation studies. It may be that the bulky adducts produced by 2-AAF are not readily detectable by the comet assay.

4.5. o-Anisidine

o-Anisidine hydrochloride is a known rodent carcinogen, inducing transitional cell carcinomas and papillomas of the urinary bladder in both sexes of rats and mice, and transitional cell carcinomas and follicular cell tumors of the renal pelvis and thyroid gland, respectively, in male rats [41]. *o*-Anisidine is oxidatively activated by peroxidase and cytochrome P-450; metabolites such as *N*-(2-methoxyphenyl) hydroxylamine covalently bind to DNA and are reactive with protein and glutathione [42,43]. In a ^{32}P-postlabeling study of treated rats, *o*-anisidine-DNA adducts were detected in urinary bladder, the primary target organ of carcinogenicity, and to a lesser extent, in the liver, kidney, and spleen [44]. In previous genetic toxicity testing, *o*-anisidine was negative in the UDS and MN assays, and positive in bacterial mutagenicity and *in vitro* chromosome aberration tests [10,12,13,45]. It was also positive in the TGR mutation test in urinary bladder, but not in liver [17,46]. In previous comet assays, *o*-anisidine tested positive in kidney and urinary bladder; the significance of positive results observed in non-target organs (lung, stomach, and colon) at only an intermediate time point (8 h, but not 3 or 24 h) following administration of a single concentration is uncertain [25]. Under the conditions of our study, *o*-anisidine was equivocal in liver and negative in stomach. Considering the target organ specificity of this chemical, this result might be considered consistent with expectations but highlights a limitation of the comet assay validation protocol that relies solely on results obtained for liver and stomach. For situations in which carcinogenicity has been noted in organs other than the liver or the gastrointestinal tract, it may be appropriate to examine additional target organ(s) using the comet assay to more completely investigate a potential genotoxic mode of action.

4.6. Sodium chloride

Sodium chloride does not induce tumors but has been shown to exert tumor-promoting activity in the glandular stomach and forestomach of chemical-initiated rats [47,48]. It tested negative in previous bacterial mutation and *in vitro* chromosomal aberration assays as well as a stomach UDS assay in rats and a bone marrow MN assay in mice [12,49,50]. Sodium chloride was included in the validation study specifically to evaluate the toxic effects of this non-genotoxic non-carcinogen in stomach in the comet assay. The results of the validation study were negative for DNA damage for both liver and stomach. Although neither the neutral diffusion assay or hedgehog tabulation suggested excessive cytotoxicity, histopathologic evaluation did reveal minimal to mild acute inflammation in a dose-dependent manner in the stomach of rats exposed to sodium chloride.

5. Conclusion

Of the five genotoxic carcinogens tested in the comet assay validation studies in our laboratory, only two — 1,2-DMH and DMN — produced significant dose-dependent increases in DNA damage. Both chemicals also produced dose-dependent increases in hedgehogs, but not LMW DNA, in the same tissue (liver) exhibiting increased DNA migration. Histopathological analysis of the liver revealed no lesions or evidence of cytotoxicity (apoptosis or necrosis) in these animals. The negative results for liver and stomach obtained for sodium arsenite, and the equivocal and negative results for liver and

stomach, respectively, obtained for *o*-anisidine, are consistent with the mode of genotoxicity and tissue specificity exhibited by these carcinogens. In contrast, given the known ability of 2-AAF, a liver carcinogen, to promote DNA bulky adduct formation, it is unclear why the comet assay often fails to definitively detect increases in DNA damage in the liver following treatment with 2-AAF, as was the case in our study. The non-genotoxic non-carcinogen, sodium chloride, was negative in both liver and stomach as would be predicted. Thus, if tissue specificity and mode of action are taken into account, the results for five out of the six comet assays conducted in our laboratory yielded results consistent with VMT expectations.

Acknowledgments

Funding

This work was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (NIEHS) under contract N01-ES-35514. Although the manuscript was reviewed by NIEHS, the statements, opinions, or conclusions contained therein do not necessarily represent the statements, opinions, or conclusions of NIEHS, NIH, or the United States government.

The authors are indebted to John Winters, Amanda Green, Patricia Allen, Jeff Davis, and Leah Zorrilla for expert technical assistance scoring comet slides, and/or critical review and statistical analyses of the data. The authors also acknowledge the supportive contributions of members of the Genetic Toxicology, Investigative Toxicology, and Necropsy programs at ILS and are grateful to Stephanie Smith-Roe Ph.D. and Raymond Tice, Ph.D. who provided critical review of the manuscript.

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Table 1

DNA damage in male rats following treatment with 2-acetylaminofluorene.[a]

Dose (mg/kg/day)	% Tail DNA		Tail length (μ)	OTM	# Hedgehogs
	Mean[a]	*p*-value	Mean	Mean	Mean
Liver					
0	0.4 ± 0.10	–	30.6 ± 0.68	0.0 ± 0.01	2.4 ± 0.60
250	1.5 ± 0.47	0.0233[b]	33.1 ± 2.33	0.2 ± 0.06	1.8 ± 0.73
500	0.8 ± 0.08	0.5930	32.0 ± 0.69	0.1 ± 0.01	2.2 ± 0.58
1000	1.0 ± 0.20	0.3395	32.2 ± 1.46	0.1 ± 0.02	1.0 ± 0.45
Trend test *p*-value	*p* = 0.3036				
EMS	14.8 ± 1.44	0.0003[c]	51.6 ± 2.17	2.4 ± 0.26	2.8 ± 0.58
Stomach					
0	13.6 ± 1.66	–	45.3 ± 0.84	2.1 ± 0.29	36.8 ± 12.07
250	18.8 ± 2.68	0.1860	46.0 ± 1.66	2.9 ± 0.49	60.2 ± 18.20
500	12.6 ± 1.60	0.9634	45.6 ± 1.85	1.9 ± 0.27	40.6 ± 6.96
1000	10.8 ± 1.63	0.6076	41.7 ± 0.92	1.5 ± 0.23	25.6 ± 7.05
Trend test *p*-value	*p* = 0.7083				
EMS	31.9 ± 2.21	<0.0001[c]	53.6 ± 1.99	6.3 ± 0.70	125.4 ± 22.27

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.025$.

Table 2

DNA damage in male rats following treatment with *N*-nitrosodimethylamine.[a]

Dose (mg/kg/day)	% Tail DNA		Tail length (μ)	OTM	# Hedgehogs
	Mean	*p*-value	Mean	Mean	Mean
Liver					
0	0.1 ± 0.01	–	29.2 ± 0.89	0.0 ± 0.00	1.0 ± 1.00
0.63	14.1 ± 1.68	<0.0001[b]	47.8 ± 1.66	2.4 ± 0.38	10.0 ± 3.36
1.25	17.5 ± 1.80	<0.0001[b]	50.7 ± 1.68	3.0 ± 0.38	28.8 ± 10.89
2.5	13.3 ± 2.18	<0.0001[b]	47.6 ± 2.78	2.1 ± 0.44	79.2 ± 23.63
Trend test *p*-value	p < 0.0001[b]				
EMS	15.3 ± 0.95	0.0003[c]	48.2 ± 0.98	2.4 ± 0.17	1.4 ± 0.93
Stomach					
0	9.5 ± 1.93	–	40.6 ± 1.68	1.4 ± 0.31	34.8 ± 12.42
0.63	11.8 ± 1.77	0.6210	43.4 ± 1.62	1.8 ± 0.32	30.4 ± 10.05
1.25	12.4 ± 1.17	0.4496	42.9 ± 1.42	2.0 ± 0.21	39.6 ± 13.42
2.5	12.5 ± 1.36	0.4263	42.1 ± 1.49	1.8 ± 0.22	53.0 ± 14.94
Trend test *p*-value	*p* = 0.2151				
EMS	30.2 ± 1.21	<0.0001[c]	54.6 ± 1.09	5.9 ± 0.28	83.0 ± 9.98

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.025$.

Table 3

DNA damage in male rats following treatment with *o*-anisidine.[a]

Dose (mg/kg/day)	% Tail DNA		Tail length (μ)	OTM	# Hedgehogs
	Mean	*p*-value	Mean	Mean	Mean
Liver					
0	0.3 ± 0.06	–	26.9 ± 0.85	0.0 ± 0.01	5.0 ± 2.10
150	0.4 ± 0.11	0.7645	28.9 ± 0.69	0.0 ± 0.01	3.8 ± 0.86
300	0.4 ± 0.15	0.7525	27.5 ± 1.08	0.0 ± 0.01	3.6 ± 0.93
600	0.9 ± 0.22	0.0182[b]	30.1 ± 1.46	0.1 ± 0.02	2.8 ± 0.66
Trend test *p*-value	*p* = 0.4242				
EMS	15.9 ± 0.81	<0.0001[c]	48.5 ± 1.07	2.6 ± 0.18	2.2 ± 0.58
Stomach					
0	14.3 ± 2.36	–	42.9 ± 1.58	2.2 ± 0.44	63.4 ± 18.16
150	12.2 ± 2.21	0.7934	43.6 ± 2.52	1.9 ± 0.51	48.2 ± 9.91
300	14.4 ± 0.91	1.0000	45.5 ± 1.92	2.1 ± 0.27	63.0 ± 11.26
600	15.9 ± 2.49	0.9601	46.3 ± 3.43	2.5 ± 0.57	46.0 ± 7.24
Trend test *p*-value	*p* = 0.9794				
EMS	35.3 ± 1.97	<0.0001[c]	56.3 ± 1.66	6.9 ± 0.51	126.4 ± 10.34

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.025$.

Table 4

DNA damage in male rats following treatment with 1,2- dimethylhydrazine dihydrochloride.[a]

Dose (mg/kg/day)	% Tail DNA		Tail length (μ)	OTM	# Hedgehogs
	Mean	*p*-value	Mean	Mean	Mean
Liver					
0	0.2 ± 0.04	–	33.2 ± 0.16	0.0 ± 0.01	0.2 ± 0.20
1.56	7.6 ± 0.26	<0.0001[b]	49.0 ± 1.27	1.4 ± 0.05	9.2 ± 1.88
3.13	13.3 ± 1.17	<0.0001[b]	55.2 ± 1.52	2.5 ± 0.35	28.6 ± 8.87
6.25	16.5 ± 1.25	<0.0001[b]	58.8 ± 1.58	3.5 ± 0.39	44.8 ± 7.50
Trend test *p*-value	$p < 0.0001$[b]				
EMS	11.6 ± 0.78	<0.0001[c]	51.0 ± 0.74	2.0 ± 0.13	1.8 ± 0.66
Stomach					
0	12.5 ± 1.56	–	44.4 ± 1.35	1.9 ± 0.27	16.0 ± 5.99
1.56	15.0 ± 1.75	0.6883	45.8 ± 1.35	2.3 ± 0.37	24.2 ± 6.94
3.13	11.7 ± 2.29	0.9856	43.8 ± 1.77	1.9 ± 0.48	36.6 ± 10.99
6.25	15.3 ± 2.13	0.6054	45.5 ± 2.43	2.2 ± 0.33	30.2 ± 8.37
Trend test *p*-value	$p = 0.7881$				
EMS	31.6 ± 1.31	<0.0001[c]	53.8 ± 2.07	6.0 ± 0.55	62.0 ± 6.99

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.025$.

Table 5

DNA damage in male rats following treatment with sodium chloride.[a]

Dose (mg/kg/day)	% Tail DNA		Tail length (μ)	OTM	# Hedgehogs
	Mean	*p*-value	Mean	Mean	Mean
Liver					
0	0.4 ± 0.16	–	27.9 ± 1.84	0.1 ± 0.01	2.8 ± 0.49
500	0.3 ± 0.10	0.6685	27.5 ± 0.39	0.0 ± 0.01	1.2 ± 0.49
1000	0.3 ± 0.06	0.5437	29.1 ± 0.85	0.0 ± 0.01	1.2 ± 0.37
2000	0.2 ± 0.06	0.3143	27.9 ± 0.99	0.0 ± 0.01	2.0 ± 0.84
Trend test *p*-value	*p* = 0.2716				
EMS	18.8 ± 0.72	<0.0001[b]	46.3 ± 0.69	2.7 ± 0.12	4.0 ± 0.71
Stomach					
0	14.1 ± 2.75	–	48.5 ± 2.56	2.4 ± 0.51	31.6 ± 5.77
500	14.7 ± 2.03	0.9949	46.2 ± 2.37	2.3 ± 0.36	37.4 ± 17.35
1000	14.3 ± 2.00	0.9998	45.9 ± 1.37	2.2 ± 0.32	26.8 ± 5.64
2000	9.1 ± 1.44	0.2527	44.0 ± 1.18	1.4 ± 0.19	31.4 ± 10.04
Trend test *p*-value	*p* = 0.9494				
EMS	33.7 ± 1.49	0.0001[b]	54.6 ± 3.01	6.3 ± 0.39	116.8 ± 31.85

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.025$.

Table 6

DNA damage in male rats following treatment with sodium arsenite.[a]

Dose (mg/kg/day)	% Tail DNA		Tail Length (μ)	OTM	# Hedgehogs
	Mean	*p*-value	Mean	Mean	Mean
Liver					
0	0.2 ± 0.04	–	32.2 ± 0.93	0.0 ± 0.01	0.0 ± 0.00
7.5	0.3 ± 0.08	0.7407	33.2 ± 0.35	0.0 ± 0.01	0.2 ± 0.20
15.0	0.2 ± 0.02	0.9977	33.0 ± 0.62	0.0 ± 0.00	0.0 ± 0.00
30.0	0.4 ± 0.10	0.1953	34.1 ± 0.57	0.1 ± 0.02	1.4 ± 0.24
Trend test *p*-value	*p* = 0.8851				
EMS	12.3 ± 1.04	0.0002[b]	52.7 ± 1.49	2.2 ± 0.25	1.2 ± 0.37
Stomach					
0	14.5 ± 2.70	–	44.1 ± 1.84	2.2 ± 0.53	39.0 ± 14.61
7.5	11.2 ± 2.55	0.6551	41.0 ± 2.93	1.6 ± 0.44	41.6 ± 18.62
15.0	11.1 ± 1.36	0.6341	42.9 ± 1.23	1.5 ± 0.24	22.0 ± 7.52
30.0	10.4 ± 2.60	0.4995	41.5 ± 1.97	1.4 ± 0.36	56.8 ± 21.43
Trend test *p*-value	*p* = 0.3318				
EMS	31.0 ± 1.78	0.0005[b]	52.5 ± 1.36	5.8 ± 0.50	102.4 ± 15.72

Abbreviations: EMS = ethyl methanesulfonate (positive control); OTM = olive tail moment.

[a] Data are presented as mean ± standard error of the mean; % tail DNA, tail length and OTM means are calculated from slide median data.

[b] Significant at $p < 0.025$.

Table 7

Results of neutral diffusion assays.[a]

Dose (mg/kg/day)	% of Cells with LMW DNA			
	Liver		Stomach	
	Mean	*p*-value	Mean	*p*-value
2-AAF				
0	3.4 ± 1.33	–	4.2 ± 1.16	–
250	3.2 ± 0.80	0.9992	16.6 ± 4.39	0.0123[b]
500	4.8 ± 2.18	0.8204	12.8 ± 2.11	0.0905
1000	1.6 ± 0.81	0.6933	5.0 ± 1.84	0.9931
Trend test *p*-value	*p* = 0.4002		*p* = 0.6113	
EMS	1.2 ± 0.73	0.1849	6.2 ± 1.46	0.3149
DMN				
0	1.8 ± 0.58	–	9.4 ± 2.38	–
0.63	1.2 ± 0.73	0.8588	6.6 ± 1.50	0.5890
1.25	1.2 ± 0.49	0.8588	6.4 ± 1.86	0.5394
2.5	0.8 ± 0.80	0.591	6.2 ± 1.53	0.4914
Trend test *p*-value	*p* = 0.5315		*p* = 0.2689	
EMS	1.6 ± 0.60	0.5915	5.2 ± 0.92	0.9310
o-Anisidine				
0	3.0 ± 0.00	–	12.2 ± 0.92	–
150	2.8 ± 1.07	0.9956	9.8 ± 1.93	0.7192
300	2.2 ± 0.80	0.8096	12.8 ± 2.87	0.9961
600	2.8 ± 0.80	0.9956	10.4 ± 3.30	0.9144
Trend test *p*-value	*p* = 0.8233		*p* = 0.8179	
EMS	2.8 ± 0.49	0.6938	6.4 ± 1.08	0.0034[c]
1,2-DMH				
0	2.0 ± 0.71	–	3.0 ± 1.30	–
1.56	0.8 ± 0.37	0.3185	2.4 ± 0.51	0.9751
3.13	1.8 ± 0.49	0.9881	4.2 ± 1.74	0.8459
6.25	1.2 ± 0.58	0.6185	2.8 ± 1.24	0.9990

Dose (mg/kg/day)	% of Cells with LMW DNA			
	Liver		Stomach	
	Mean	*p*-value	Mean	*p*-value
Trend test *p*-value	*p* = 0.8012		*p* = 0.5164	
EMS	2.6 ± 0.93	0.3104	4.6 ± 0.81	0.1640
Sodium chloride				
0	2.4 ± 0.93	–	7.0 ± 1.41	–
500	5.0 ± 2.49	0.4565	10.2 ± 2.33	0.5187
1000	2.8 ± 0.97	0.9945	10.4 ± 2.16	0.4732
2000	4.6 ± 0.51	0.5801	12.6 ± 1.63	0.1346
Trend test *p*-value	*p* = 0.4739		*p* = 0.0576	
EMS	4.0 ± 0.77	0.2220	11.8 ± 3.60	0.2495
Sodium arsenite				
0	1.2 ± 0.58	–	2.8 ± 1.32	–
7.5	2.6 ± 1.03	0.4857	3.2 ± 1.39	0.9897
15.0	2.2 ± 0.92	0.7136	2.4 ± 0.75	0.9897
30.0	2.8 ± 0.58	0.3831	3.6 ± 1.08	0.9290
Trend test *p*-value	*p* = 0.3916		*p* = 0.8108	
EMS	1.6 ± 0.68	0.3333	3.6 ± 0.24	0.2906

Abbreviations: EMS = ethyl methanesulfonate (positive control); LMW = low molecular weight.

[a] Data are presented as mean ± standard error of the mean.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.025$.