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Investigation of sodium arsenite, thioacetamide, and diethanolamine in the alkaline comet assay: Part of the JaCVAM comet validation exercise

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ABSTRACT

As part of the Japanese Center for the Validation of Alternative Methods (JaCVAM)-initiative international validation study of the *in vivo* rat alkaline comet assay (comet assay), we examined sodium arsenite, thioacetamide, and diethanolamine. Using the JaCVAM approved study protocol version 14.2, each chemical was tested in male rats up to maximum tolerated dose levels and DNA damage in the liver and stomach was assessed approximately 3 h after the final administration by gavage. Histopathology assessments of liver and stomach sections from the same animals were also examined for evidence of cytotoxicity or necrosis. No evidence of DNA damage was observed in the stomach of animals treated with sodium arsenite at 7.5, 15, or 30 mg/kg/day. However, equivocal findings were found in the liver, where increases in DNA migration were observed in two independent experiments, but not in all treated animals and not at the same dose levels. Thioacetamide caused an increase in DNA migration in the stomach of rats treated at 19, 38, and 75 mg/kg/day, but not in the liver, despite evidence of marked hepatotoxicity following histopathology assessments. No evidence of DNA damage was observed in the stomach or liver of animals treated with diethanolamine at 175, 350, or 700 mg/kg/day.

1. Introduction

The *in vivo* rodent alkaline comet assay (comet assay) is used worldwide for detecting DNA damage induced by genotoxic agents. The assay is used for investigating the genotoxic potential of test chemicals, and is expected to be used as a second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance [1] after the *in vivo* micronucleus assay with bone marrow and/or peripheral blood. The comet assay has been discussed at the meetings of the International Workshop on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published [2–4]. The assay, however, has not been validated formally with a standardized study protocol. 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 Centre for the Validation of Alternative Methods (ECVAM), and the Mammalian Mutagenicity Study Group (MMS)/Japanese Environmental Mutagen Society (JEMS). The purpose of this validation study was to evaluate the ability of the *in vivo* comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity. The validation study results have been submitted to the Organization for Economic Co-operation and Development (OECD) for establishment of a test guideline.

As a part of the 2nd step of 4th phase international validation study, we examined sodium arsenite, thioacetamide, and diethanolamine under coded test chemical conditions. The identity of each chemical was revealed by the Validation Management Team (VMT) after the comet data had been reported. The existing genotoxicity data for these chemicals has been summarized by the VMT [5] or reported elsewhere [6–10].

2. Materials and methods

The study was conducted in accordance with the validation study protocol version 14.2 [11]. The following are the actual testing conditions employed.

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2.1. Test chemicals

The three test chemicals were supplied by the JaCVAM Validation Management Team (VMT) and at the time of testing were identified solely by their VMT code.

Sodium arsenite (CAS 7784-46-5; VMT code A4129), thioacetamide (CAS 62-55-5; VMT code A4220), and diethanolamine (CAS 111-42-2; VMT code A4221). All three chemicals were stored at room temperature, protected from light. Formulations for animal dosing were prepared by dissolving each chemical in physiological saline at concentrations necessary to achieve the final dose level. Physiological saline also acted as the vehicle control for dosing negative control animals. Ethyl methanesulfonate (EMS; CAS 62-50-0, supplied by Sigma–Aldrich, UK) was used as the positive control. It was formulated in physiological saline at a concentration of 20 mg/mL. All dose formulations, including the vehicle and positive controls, were prepared daily and used within 2 h of preparation. Ethidium bromide (supplied by Sigma–Aldrich, UK), prepared in water at 2 μg/mL was used for staining of all comet slides. Hank's balanced salt solution (HBSS), used to prepare the mincing solution, was supplied by Invitrogen, Paisley, UK. All other standard chemicals and reagents were supplied by Sigma–Aldrich or equivalent suppliers.

2.2. Animals

Out-bred young adult male Sprague–Dawley Crl:CD® (SD) rats were obtained from Charles River (UK) Ltd., Margate, UK. The only exception to this was the rats used for the thioacetamide comet experiment, which were supplied by Harlan UK Ltd., Oxon, UK. Animals were 7–8 weeks old and in the region of 200–250 g at the time of dosing in the comet assay. They were randomly allocated to cages on arrival from the supplier and were housed in solid bottom cages at a density of 3 per cage (dose range-finding) or 6 per cage (comet experiments). Animals were housed in rooms air-conditioned to provide 15–20 air changes/h with temperature and relative humidity maintained at 20–24 °C and 45–65%, respectively. Fluorescent lighting was controlled automatically to give a cycle of 12 h light and 12 h dark. Animals were given SQC Rat and Mouse Maintenance Diet No 1, Expanded (Special Diets Services Ltd., Witham, UK) and municipal supply water (in water bottles) *ad libitum*.

2.3. Animal treatment

Animals were dosed orally by gavage using dose volumes of 10 mL/kg. The highest dose tested for sodium arsenite, thioacetamide, and diethanolamine was determined to be the maximum tolerated dose (MTD) during dose range-finding assessments in groups of 3 rats, using the same dosing regimen as the comet experiments. The lower dose levels were equivalent to 25% MTD or 50% MTD. Groups of 6 male rats were treated with either saline (vehicle control), 1 of 3 dose levels of sodium arsenite, thioacetamide or diethanolamine or 200 mg/kg EMS. Animals were dosed at 0, 24, and 45 h, with necropsy and tissue sampling performed at 48 h (*i.e.*, 3 h after the final administration). Animals were dosed and sampled in ascending dose order (*i.e.*, vehicle control, low, intermediate then high dose) with positive controls treated and sampled last.

2.4. Comet assay

Single experiments were conducted with thioacetamide and diethanolamine; due to equivocal findings with sodium arsenite, a second experiment was conducted, with comet analysis of liver only. Animals were killed by terminal isoflurane anaesthesia ensured by exsanguination. Animals were examined internally for signs of unusual coloration or abnormalities to organs/tissues. Liver and stomach were removed from each animal.

2.4.1. Liver cell preparation

A section of the left lateral lobe of the liver was removed, placed on ice in mincing solution (HBSS, 20 mmol/L ethylenediaminetetraacetic acid [EDTA] disodium salt, pH 7.0–7.5, 10% dimethylsulphoxide [DMSO]) prior to being cut into small pieces in fresh, cold mincing solution. The pieces of liver were then pushed through bolting cloth (pore size of 150 μm) with approximately 4 mL of mincing solution to produce single cell suspensions.

2.4.2. Stomach cell preparation

The stomach was cut in half, longitudinally, and a section of the fundic region was washed in cold mincing solution and then incubated on ice for 15 min, covered in fresh mincing solution. After incubation the samples were removed and placed in 200 μL of fresh mincing solution. Cells were gently scraped from the inside of the stomach using the back of a scalpel blade to produce single cell suspensions. Tissue samples were held on ice and processed to cell suspensions within 1 h of necropsy. Cell suspensions were held on ice and used to prepare slides within 1 h of cell preparation.

2.4.3. Slide processing and comet scoring

A 10 μL aliquot of each single cell suspension was added to 0.5% low melting point agarose and 100 μL of cell suspension/agarose mix was subsequently placed on to a slide, previously coated with normal melting point agarose. The slides were then cover-slipped and gelled over ice. Once gelled, the coverslips were removed and slides placed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO). Lysis was performed overnight at 1–10 °C, protected from light. Following lysis, slides were washed briefly in purified water and transferred to electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 or 30 min for the stomach or liver slides, respectively (DNA unwinding), prior to electrophoresis in the same buffer at 0.7 V/cm for 20 or 40 min, for stomach or liver slides, respectively. Immediately after electrophoresis, slides were neutralized in 0.4 M tris pH 7.0 (3 × 5 min washes) and then dehydrated in absolute ethanol for 5 min. Dehydrated slides were dried and stored at room temperature prior to scoring. Slides were stained with 100 μL of 2 μg/mL ethidium bromide and coverslipped, prior to scoring by fluorescence microscopy, ×200 magnification, 510–560 nm excitation filter, 590 nm barrier filter using Perceptive Instruments' (Suffolk, UK) 'Comet Assay IV' image analysis system. Measurements of tail intensity (TI; equivalent to the percentage of DNA in the tail), Olive tail moment (TM), and tail length (TL) were obtained from 100 cells per animal per tissue, usually achieved by scoring 50 cells from two slides. Highly damaged cells ("hedgehogs") were excluded from comet analysis but the number present in a 100 cells (assessed per slide scored) was recorded. Prior to scoring, all slides were coded so the analyst could not identify the treatment conditions and ensured blinded scoring of the comets.

2.5. Histopathology

The remaining left lateral lobe of the liver, the entire right median lobe of the liver, and the entire left longitudinal section of the stomach (with duodenum) were preserved at room temperature in neutral buffered formalin: Preserved samples were embedded in wax blocks and stored at room temperature. Blocks were sectioned at a nominal 5 μm, stained with haematoxylin and eosin and examined by a pathologist.

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