Regulatory Toxicology and Pharmacology 58 (2010) 145–154

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

# Evaluation of multi-organ DNA damage by comet assay from 28 days repeated dose oral toxicity test in mice: A practical approach for test integration in regulatory toxicity testing

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ARTICLE INFO

*Article history:*
Received 1 October 2009
Available online 19 May 2010

*Keywords:*
Test integration
DNA damage
Comet assay
Micronucleus assay
Methotrexate
Mice

ABSTRACT

The use of comet assay is not new in the evaluation of genotoxic potential of different agents; however, its broad use in product safety for regulatory testing is a relatively new approach. The present study was aimed to integrate genotoxicity tests (micronucleus and comet assay) in 28 days repeated dose oral toxicity of methotrexate (MTX) in mice. MTX was administered at the dose of 0.5, 1 and 2 mg/kg per oral repeatedly for 28 days in mice. The endpoints of evaluation for routine toxicity testing included body weight, organ weight, food intake, water intake, hematology and histology, while for the genotoxicity testing micronucleus and comet assay were used. There were no significant changes in food intake, water intake and organ weight; however, the body weight significantly decreased at the highest dose of MTX treatment as compared to control group. Histological data revealed the morphological alterations in the liver and lung cells at the highest dose of MTX treatment. Micronucleus assay results indicated that the highest dose of MTX led to significant increase in MNERTs/1000ERTs ($P < 0.001$) as compared to control group. Further, percentage of reticulocytes (% RETs) was significantly decreased at the highest dose of MTX as compared to control group. Comet assay results indicate significant DNA damage in different organs induced by MTX as compared to control group. The results of the present study successfully demonstrates the integration of genotoxicity tests using comet and micronucleus assay in 28 days repeated dose oral toxicity test. Integration of genotoxicity test with routine toxicity test would reduce the cost of additional animals, test item and provide further information at an early stage of product development.


## 1. Introduction

Test integration is a novel effort in the repeated-dose toxicity testing, but least exploration has been done in this area of scientific research. Genetic toxicity screening plays a vital role in the product development and marketing of new chemicals for pharmaceutical, agricultural, industrial and consumer use. MacGregor et al. recommended the integration of cytogenetic analysis for DNA damage in the repeated dose toxicological testing for industrial setting (MacGregor et al., 1995). Krishna et al. enriched the concept of test integration in as early as 1998 and advocated that the micronucleus (MN) test can be successfully carried out along with routine 2–4 weeks toxicokinetic studies. Successful integration of genotoxicity test into the routine toxicology testing in the early stage of product development has several advantages (Krishna et al., 1998). Efforts are being made by the International Workshop on Genotoxicity Test Procedures (IWGTP) to design the practical aspects of protocol including repeated dose treatments, integration with other routine toxicity testing and automated scoring of *in vivo* rodent erythrocytes MN assay (Hayashi et al., 2000). The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation emphasizes that the new technologies require a shift on the way in which safety assessment is conducted for different chemicals. Further, it has been realized that the Integrated Testing Strategies (ITSs) are already in use for toxicological evaluation, but the concept of their integration and application in a regulatory setting have yet to be fully implemented (Hoffmann et al., 2008). The Joint Research Centre of the European Commission estimated that the highest number of *in vivo* genotoxicity tests are essential to evaluate the chemicals under REACH program (Van der Jagt et al., 2004). In a recent workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) held from 24 to 25 June 2008, it was emphasized that integration of

*Abbreviations:* MTX, methotrexate; MN, micronucleus; IWGTP, International Workshop on Genotoxicity Test Procedures; REACH, Registration Evaluation Authorization Restriction of Chemicals; MDA, malondialdehyde; GSH, glutathione; RETs, reticulocytes; NCEs, normochromatic erythrocytes; ERTs, erythrocytes.

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doi:10.1016/j.yrtph.2010.05.004

genotoxicity endpoints into the repeated-dose toxicity study in a scientifically justified manner can reduce the number of animals used in toxicity testing (Pfuhler et al., 2009).

The comet assay is a single cell gel electrophoresis technique for detecting DNA damage at single cell level. The most important advantage is that the DNA lesions can be measured in any organ, regardless of the extent of mitotic activity (Tsuda et al., 2000). The expert panel at the IWGTP held in Washington, DC, March 25–26, 1999 reached a consensus that the alkaline (pH >13) version of the comet assay is suitable for identifying agents with genotoxic activity (Tice et al., 2000). Sekihashi et al., compared the effect of numerous model compounds administered through intraperitoneal route and oral gavage and concluded that the results obtained with both the routes are acceptable for the comet assay (Sekihashi et al., 2001). Sekihashi et al., further reported that 49 out of 54 rodent carcinogens that do not induce MN, were found positive in the comet assay, suggesting that the comet assay can be used as a further *in vivo* test apart from the cytogenetic assays in rodent hematopoietic cells (Sekihashi et al., 2002). The comet assay is now widely used in regulatory, mechanistic and biomonitoring studies in a range of *in vitro* and *in vivo* test systems (Lovell and Omori, 2008). Kirkland and Speit emphasized that when an *in vitro* positive results are obtained, the most appropriate follow-up testing in *in vivo* should be the transgenic mutation (TG) and comet assay in a regulatory testing strategies than the unscheduled DNA synthesis (Kirkland and Speit, 2008). In the present experiment an attempt has been made to evaluate the systemic toxicity of MTX in mice employing the parameters such as body weight, feed intake, water intake, organ weight, hematology, oxidative stress as well as tissue histology. Further, efforts have been made to integrate both MN as well as comet assay into the repeated dose oral toxicity study in mice for regulatory acceptance.

## 2. Materials and methods

### 2.1. Animals

All animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), India. Experiments were performed on male Swiss albino mice (weight range in between 22 and 25 g) procured from Institute's Central Animal Facility and kept at controlled environmental conditions with room temperature (22 ± 2 °C), humidity (50 ± 10%) and automatic controlled dark and light cycle (0600–1800 h). Six animals were used in each group. Standard laboratory animal feed (purchased from Tetragone Chemi Pvt. Ltd., Bangaluru, India) and water (aquapure) were provided *ad libitum*. Animals were acclimatized to the experimental conditions for a period of 1 week before the initiation of the experiment.

### 2.2. Chemicals

MTX (CAS 59-05-2) was obtained as a gift sample with manufacturing batch No: CK0705002 from GlaxoSmithKline Pharmaceuticals Limited, Mumbai, India. SYBR green-I (CAS 163795-75-3) and Hematoxylin and Eosin (H&E) were purchased from Sigma–Aldrich, USA. Normal melting point agarose (NMA), low melting point agarose (LMA), Hank's balanced salt solution (HBSS), dimethylsulphoxide (DMSO), Triton X-100, and ethylenediamine-tetraacetic acid (EDTA) were obtained from Hi-media Laboratories Ltd., Mumbai, India.

### 2.3. Dose selection, chemical preparation and animal treatment

The present doses of MTX (0.5, 1 and 2 mg/kg) were selected based on our own experiences and the studies carried out by Padmanabhan et al. in the same laboratory, which show the cytotoxic and genotoxic effect of MTX in germ cells of mice and its subsequent protection by the intervention of folic and folinic acid (Padmanabhan et al., 2008, 2009). MTX was freshly prepared in 0.1 M sodium bicarbonate ($NaHCO_3$) and given at the doses of 0.5,1 and 2 mg/kg per orally. Animals were divided in group I to IV ($n = 6$) and they received vehicle, 0.5, 1 and 2 mg/kg of MTX, respectively. The volume administered was 10 ml/kg per day for 28 days.

### 2.4. Animal observations

All animals were observed twice daily for any toxicity, morbidity and mortality. Body weights were recorded immediately pretest, weekly and on the day prior to sacrifice. On day 28, animals were fasted overnight and on day 29, animals were sacrificed.

### 2.5. Hematology

Blood samples were collected by cardiac puncture at the time of sacrifice for hematological analysis. The following parameters for hematology were analysed using a MELET SCHLOESING LABORATORIES (MS9-5 France) hematology analyzer: hematocrit (Hct), hemoglobin concentration (Hb), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet count (Plt), white blood cell (WBC) and red blood cell (RBC) count.

### 2.6. Histopathology

Liver and lung were isolated for histopathology. Tissues were fixed in 10% formalin solution. Paraffin blocks were prepared after completing the tissue processing in different grades of alcohol and xylene. Sections (5 μm) were prepared from paraffin blocks using microtome, stained with hematoxylin and eosin. Images were taken using OLYMPUS camera connected to the microscope to examine gross cellular damage.

### 2.7. Estimation of oxidative stress markers malondialdehyde (MDA) and glutathione (GSH) level in liver and brain

MDA level in the liver and brain was measured according to the method described by Ohkawa et al. (1979). Further the liver and brain tissue homogenate were used for the estimation of glutathione (GSH) content as described by Moron et al. (1979). Protein content in tissue homogenate supernatant was determined as described by Lowry et al. (1951).

### 2.8. Peripheral blood micronucleus assay

Peripheral blood smear was prepared as described by Holden et al. (1997) with some modifications (Holden et al., 1997; Jena and Bhunya, 1994). Blood was collected from tail tip before the sacrifice of the animal and the blood smear was prepared on pre-cleaned slides. The smear was allowed to dry at room temperature and fixed in absolute methanol for 5 min. After fixation slides were stained with acridine orange (AO) and washed twice with phosphate buffer (pH 6.8) (Hayashi et al., 1994). Slides were observed under oil immersion objective (100×) using an Olympus fluorescent microscope (Model BX 51) connected to digital photomicrograph software (OLYSIA BioReport, 2001). In total 1000 cells were scored for the incidence of MN in the erythrocytes, which includes both immature reticulocytes (RETs) as well as mature normocromatic erythrocytes (NCEs). Reticulocytes stained orange red and can be distinctly identified from the matured normochromatic erythrocytes (NCEs), which stained green with acridine orange.