Safety Testing with the *In Vitro* Comet Assay: Critical Factors in Protocol Design and Data Interpretation

Marie Vasquez and Nicole Dewhurst

Helix3 Inc., Morrisville, North Carolina

HELIX3 INC.

ABSTRACT

As the *in vivo* comet assay gains popularity and acceptance for use in regulated safety testing, the directive by the EU to reduce animal use in testing has encouraged laboratories to evaluate the ability of the comet assay to screen and test compounds *in vitro*. However, published guidelines for using the *in vitro* comet assay in genetic toxicology testing are based on data generated from known carcinogens. Meanwhile, other regulated *in vitro* assays requiring cell division (e.g., chromosomal aberration assay) are often used to determine the experimental conditions for the *in vitro* comet assay despite the different sample time and endpoint being measured. As the pharmaceutical and chemical industries generate new and complex classes of compounds, it is becoming more and more critical to ensure that the appropriate protocol design for testing and evaluating the true safety risk of compounds with the *in vitro* comet assay is used. To demonstrate how sample time and the type of damage induced can change or influence the interpretation of *in vitro* comet assay results, data from time course experiments on different classes of genotoxins (e.g., strand breakers, crosslinkers) are presented.

INTRODUCTION

To provide the most comprehensive means for interpreting *in vitro* comet assay study data, Helix3 incorporates into each study and for each comet sample a 5,6-Carboxyfluorescein diacetate and Ethidium bromide (CFDA/EtBr) dual stain viability assessment, the LMW DNA diffusion assay, and the determination of cell concentration. This study was conducted to demonstrate how the additional information obtained with these methods can enhance the interpretation of *in vitro* comet assay data for test compounds. To provide a mechanistic pathway profile for different classes of genotoxins, CHO-K1 cells were exposed to Formaldehyde (FA), a protein-DNA crosslinker, Mitomycin C (MMC), a DNA-DNA crosslinker, and Ethyl methanesulfonate (EMS), a strand breaker. Cultures were sampled at 1, 3, 6, and 24 hours after dosing.

METHODS AND MATERIALS

Cell Exposure

Chinese Hampster Ovary (CHO-K1) cells were obtained from ATCC and maintained in complete medium (Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate) with 10% fetal bovine serum (FBS) and at 37±1°C with 5±1% CO_2 in air.

Duplicate cultures were exposed in the absence of metabolic activation to EMS, FA or MMC and DMSO (vehicle control) for 1, 3, 6 and 24 hours at 37±1°C with 5±1% CO2 in air.

Doses were administered in a dose volume that was 1% of the total culture volume and 16-24 hours after culturing.

Comet Sample Processing and Analysis

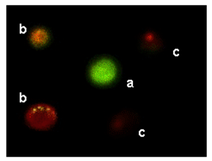
After dosing and at each sample time, cultures were washed, harvested and maintained on ice until processed further. Viabilities and cell concentrations for each harvested culture were determined. Comet sample processing was performed; based on the cell concentration, the volume of each comet sample was adjusted as necessary with mincing solution. Comet slides were prepared and processed using the standard Helix3 protocol for the alkaline version of the comet assay and LMW diffusion assay (1). To enhance the detection of decreases (crosslinks) as well as increases (strand breaks) in DNA migration, comet samples were electrophoresed at room temperature.

METHODS & MATERIALS (CONT.)

Viability Assessments

Viabilities as measured by metabolic competency were determined using a 5,6-Carboxyflourescein diacetate/Ethidium Bromide (CFDA/EtBr) dual stain where the presence of CFDA fluorescence indicates metabolically active (viable) cells regardless of cell membrane integrity and EtBr fluorescence in the absence of CFDA fluorescence indicates dead cells.

Figure 1: CHO-K1 Cells Stained with CFDA/EtBr Dual Stain

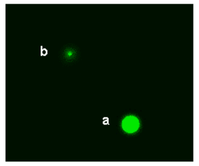


(a) Viable; (b) Compromised; (c) Dead

Cytotoxicity Assessments

To assess for cytotoxicity, one replicate comet slide was removed from lysis after 1 hour and neutralized without undergoing electrophoresis. The neutralized slides were then dipped in alcohol and air dried. Air dried slides were stained with SYBR Gold™ (Molecular Probes) and 100 cells per slides were scored visually. Each cell scored was classified using the following criteria: Type I = Mostly condensed DNA with little no diffusion; Type II = Mostly diffused DNA with little or no condensed DNA. The percentage of Type II cells is considered representative of cytotoxicity in the cell population sampled.

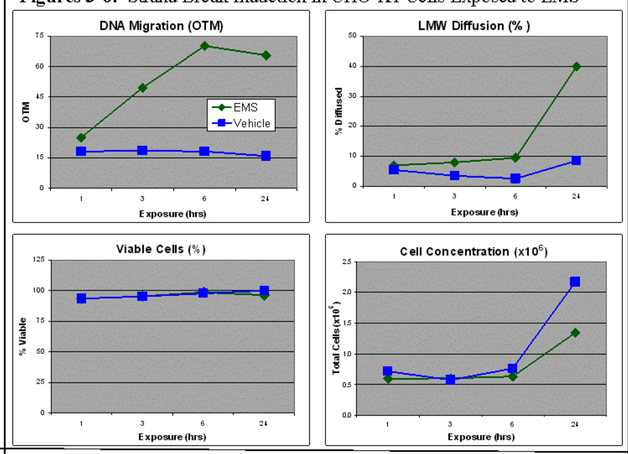
Figure 2: LMW DNA Diffusion Assay



(a) Type I (b) Type II

RESULTS

Figures 3-6: Strand Break Induction in CHO-K1 Cells Exposed to EMS

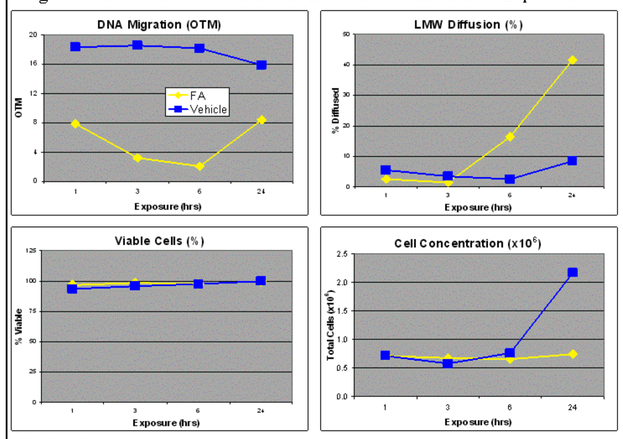


RESULTS (CONT.)

EMS Results

A significant increase in DNA migration indicative of strand breaks/alkali labile sites was detected at 3, 6 and 24 hours after the start of exposure to EMS with the maximum effect detected at 6 hours. At the same time, the lack of a significant increase in LMW DNA diffusion and the lack of a significant decrease in metabolic competency or in cell concentration indicate that the increase in DNA migration detected at 3-6 hours was most likely due to genotoxicity and not cytotoxicity. Therefore, the optimal sample time for detecting strand breaks/alkali labile sites in CHO-K1 cells exposed to EMS would be 3-6 hours after dosing.

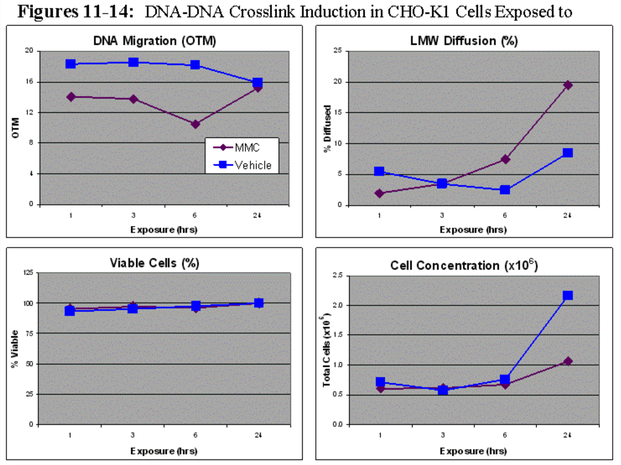
Figures 7-10: Protein-DNA Crosslink Induction in CHO-K1 Cells Exposed to FA



FA Results

A significant decrease in DNA migration indicative of protein-DNA crosslinks was detected at 1, 3, 6 and 24 hours after the start of exposure to FA with the maximum effect detected at 6 hours. This presence of protein-DNA crosslinks could be further verified by a significant decrease in LMW DNA diffusion detected 1 hour after the start of exposure when compared to the concurrent vehicle control. However, a significant increase in LMW DNA diffusion and a decrease in cell concentration at 24 hours after exposure indicates that incomplete excision repair and/or cell cycle arrest may have a confounding effect on detecting genotoxicity after 6 hours. Therefore, the optimal sample time for detecting protein-DNA crosslinks in CHO-K1 cells exposed to FA would be 1 hour after dosing.

Figures 11-14: DNA-DNA Crosslink Induction in CHO-K1 Cells Exposed to



RESULTS (CONT.)

MMC Results

A significant decrease in DNA migration indicative of DNA-DNA crosslinks was detected at 1, 3, and 6 hours after the start of exposure to MMC with the maximum effect detected at 6 hours. This presence of DNA-DNA crosslinks could be further verified by a significant decrease in LMW DNA diffusion detected 1 hour after the start of exposure when compared to the concurrent vehicle control. However, a significant increase in LMW DNA diffusion as well as in DNA migration to baseline levels at 24 hours after exposure indicates that incomplete excision repair and/or cell cycle arrest may have a confounding effect on detecting genotoxicity after 6 hours. An earlier experiment (data not presented) including the same sample times as well as a 0.5 hour sample time verified the results of this experiment and detected decreases in DNA migration and LMW DNA diffusion as early as 0.5 hour after dosing. Therefore, the optimal sample time for detecting DNA-DNA crosslinks in CHO-K1 cells exposed to MMC would be 0.5-1 hour after dosing.

CONCLUSIONS

With the constant development of new classes of compounds that can induce DNA damage in the form of strand breaks, alkali labile sites, and/or crosslinks, the comet assay can be a powerful tool for safety testing of compounds. However, for compounds with unknown or different metabolic pathways and/or that may include mechanisms such as cell cycle inhibition, it is not sufficient to follow standard *in vitro* comet assay protocols developed and based on the mechanisms of known strong genotoxins and/or based on comparative genetox studies (e.g., chromosomal aberrations) and on published data. And since the form of DNA damage (strand breaks or crosslinks), cytotoxicity, and/or excision repair can influence DNA migration patterns by either increasing or decreasing DNA migration, including only single measurements of cell viability (e.g., trypan blue exclusion, mitotic index) may result in the loss of information critical to the interpretation of the comet assay data.

Therefore, *in vitro* comet assay safety protocols should include several sample times and multiple measurements of cell health including but not limited to measurements of metabolic competency (viability), LMW DNA diffusion (apoptosis and necrosis), and cell concentration (growth). Combined with DNA migration data, these cell health measurements provide important information about cellular events (e.g., excision repair, cytotoxicity) that influence DNA migration and that are critical to the true safety assessment of test compounds using comet (2).

REFERENCES

1. Vasquez, M. and S. Pfuhler (2006) Effects of cytotoxicity on the interpretation of in vivo comet assay data. Environ. Mo. Mutagen Poster Presentation.
2. Vasquez, M. (2007) Cytotoxicity and its impact on the *in vivo* comet assay. Environ. Mo. Mutagen Poster Presentation.