The establishment of the in vitro Comet assay using six genotoxic compounds as positive controls

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INTRODUCTION

• The Comet assay is a sensitive & rapid method to measure DNA damage
• We have used the in vitro Comet assay & 6 positive control genotoxic compounds to determine DNA damage by:
  1. The Alkaline Comet assay which detects DNA strand breaks, alkali-labile sites (ALS) & DNA-DNA/DNA-protein cross linking
  2. The Modified Alkaline Comet assay which identifies oxidised purine & pyrimidine lesions by using formamidopyrimidine DNA glycosylase (FPG) & endonuclease III (ENDO III) repair enzymes, respectively
• A published Statistical method was utilised to evaluate the data

RESULTS

Alkaline Comet assay

Etoposide, MMS, EMS, H2O2 & NNKoAc treatment

Cell viability was >95% after all treatments

250 μM etoposide, 750 μM MMS, 60 mM EMS, 400 mM H2O2 & 150 μM NNKOAc induced 54.54 ± 29.40%, 41.87 ± 16.74%, 70.03 ± 11.40%, 71.92 ± 21.87% or 72.19 ± 12.32% DNA damage, respectively (Fig. 2)

Responses were significant (p<0.05) when compared to respect to vehicle control

Modified Alkaline Comet assay

Potassium Bromate (KBrO3)

Cell viability after 2.0 mM & 10.0 mM KBrO3 treatment was >98%

Minimal DNA damage was induced by 2.0 mM or 10.0 mM KBrO3 in END3 induced 70.73 ± 16.72% & 63.63 ± 25.68% oxidative DNA damage, respectively (Fig. 3)

CONCLUSIONS

1. We have developed optimal exposure conditions & positive response to all 6 genotoxic compounds in NCI-H292 cells
2. A published parametric analysis approach has enabled appropriate, meaningful interrogation & evaluation of the data

REFERENCES


METHODS

Comet assay method

Cell culture

NCI-H292 cells were supplied by ATCC (http://www.nci.atcc.org)

The day before Comet analysis, cells were seeded in 6-well plates at 1.2x10⁶cells/well

Alkaline Comet assay: Confluent H292 cells were incubated for 1 hour at 37°C with etoposide (250 μM), methyl methanesulfonate (MMS, 750 μM), ethyl methanesulfonate (EMS, 60 mM), hydrogen peroxide (H2O2, 400 μM), acetoxyethyl nitrosamine-1-(3-pyridyl)-1-butanoate (NNKOAc, 150 μM) vehicle (0.5% DMSO or 1 x PBS)

Modified Comet assay: Confluent H292 cells were incubated for 1 hour at 37°C with two concentrations of potassium bromate (KBrO3, 2.0 mM or 10.0 mM) or vehicle (1 x PBS)

Alkaline & Modified Comet assay procedure

Cell sample preparation: ~20,000 cells were resuspended in 0.6% low melting-point agarose at 37°C & placed on Superfrost Plus pre-coated with 1.0% collagen & 1.5% normal melting-point agarose

Lysis: Slides were placed in Lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma, 0.2 M NaOH, 10% DMSO & 1% Triton X-100, pH 10) for 24h at 5°C ± 3

Wash: Slides were rinsed (3x 5 min) with Enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8) at room temperature (RT)

Enzyme incubation (Modified Alkaline Comet assay only): Slides were incubated with FPG (New England BioLabs®) or ENDO III (New England BioLabs®) diluted 1:5,000 or 1:10,000, respectively, in Enzyme reaction buffer for 45 min at 37°C

Alkaline unwinding: Slides incubated with Electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) within the electrophoresis tank (Thistle Scientific) for 5 min at 5°C ± 3

Electrophoresis: 20 min at 25 constant volts in 1.0L Electrophoresis buffer at 5°C ± 3

Neutralisation: ~3.5 ml of Neutralisation buffer (0.4 M Trizma, pH 7.5) was placed on each slide

Fixation: Slides were air dried for approximately 24h at RT

Comet visualisation: 20 μL of VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories Inc.) was applied to slides & nuclei visualised using 20x magnification

Determination of cell viability

Cell viability was determined by trypan blue dye exclusion using an automated Vi-CELL® cell viability analyser (Beckman-Coulter)

Statistical analysis

~100 cells per slide were assessed. DNA damage was determined using Comet assay IV image analysis software & percentage of tail intensity (TI) recorded

Mean & standard deviation (SD) of TI were calculated

Data were analysed by using a published parametric statistical analysis approach (Fig. 1)

Figure 1: Parametric statistical analysis flow diagram, based on Bright et al. 2011

REFERENCES


Figure 2: A: DNA damage in H292 cells after 250 μM etoposide, 750 μM MMS, 60 mM EMS, 400 mM H2O2 & 150 μM NNKOAc treatment. A-E: Bar charts (Mean ± SD of TI) & F-J: Scatter plots (Individual log-transformed TI). *p<0.05 when compared to vehicle.

Figure 3: A: Strand breaks & oxidative DNA damage in H292 cells following incubation with 2.0 or 10.0 mM KBrO3 & subsequent incubation with FPG or ENDO III. A: Bar charts (Mean ± SD of TI), B: Scatter plots (Individual log-transformed TI). *p<0.05 when compared to vehicle.