Determination of the optimal protocol for single-cell gel electrophoresis assay (COMET assay) in cultured NCI-H292 human mucoepidermoid pulmonary carcinoma cells

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INTRODUCTION

- **The single-cell gel electrophoresis assay (COMET assay) is a sensitive and rapid method to measure DNA damage and identify potential genotoxic compounds[1-2]**
- **We have established optimal methods to determine DNA damage by:**
  1. **The Alkaline COMET assay which detects DNA strand breaks, alkali-labile sites (ALS) and DNA-DNA/DNA-protein cross linking**
  2. **The Modified Alkaline COMET assay which identifies oxidised purine and pyrimidine lesions by using formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (ENDO III) repair enzymes, respectively**

METHODS

Evaluation of published COMET assay methods

- Published methods were reviewed and COMET assay performed[1-5] **(Table 1 & Fig. 1)**
- Protocols were modified to establish optimal and reproducible methods for the Alkaline COMET assay and the Modified Alkaline COMET assay **(Table 1 & Fig. 1)**

Table 1: Stages investigated to optimise COMET methods

Step	Published methods[1-5]	Vivotecnia method	Rationale
1. Slide preparation	Normal slides	Superfrost slides	To avoid agarose detachment during *Electrophoresis - step 8*
	1.0% agarose	1.5% agarose	
	N/A	1.0% collagen	
2. Cell culture	24 well plates	6-well plates (high number of cells/well)	Sufficient cells for automatic counting
	Neubauer chamber	Automatic cell counter	Decrease the time to *Lysis – step 4*
3. Cell sample preparation	10,000 cells/slide	20,000 cells/slide	Decrease COMET scoring time
	Third agarose layer	Remove third agarose layer	Decrease the time to *Lysis – step 4*
	5-7 min cooling	2 min using pre-cooled metallic plate	Reduce agarose cooling time
4. Lysis	~1 hour	~24 hours	Ensure full cellular lysis
5. Wash	Tris solution	Enzyme reaction buffer	Improve removal of detergents and salts
6. Alkali unwinding	pH ~13	pH >13	Maximize detection of DNA alkali-labile sites and strand breaks
	20 min	5 min	
7. Enzyme incubation*	45 min	45 min	N/A
8. Electrophoresis	Conventional electrophoresis tank	COMET electrophoresis tank (Thistle Scientific)	Reduce levels of basal DNA damage and protect against UV degradation
9. Neutralisation	~1.5 mL Neutralisation buffer/slide	~3.5 mL Neutralisation buffer/slide	Effective removal of Electrophoresis buffer
10. Fixation	100% ethanol	Air-dry for ~24h	Improve staining
11. COMET visualisation	Ethidium bromide	DAPI	Avoid use of teratogenic compounds
	10x magnification	20x magnification	Improve COMET visualisation

(Steps 2–3, bracketed: ~20 min)

* Stage 7 only performed for the Modified Alkaline Comet assay. N/A: not applicable

Vivotecnia method for the COMET assay

Cell culture

- NCI-H292 cells were supplied by ATCC (http://www.lgcstandards-atcc.org)
- The day before COMET analysis, cells were seeded in 6-well plates at 1.2×10^5 cells/well

Alkaline COMET assay

- Confluent H292 cells were incubated for 1 hour at 37ºC with Etoposide (250 µM), Methyl methanesulfonate (MMS; 750 µM), Hydrogen peroxide (400 µM; H_2O_2) or vehicle (0.5% DMSO or 1 x PBS)
- COMET assay was performed as described in **Table 1**

Modified Alkaline COMET assay

- Confluent H292 cells were incubated for 1 hour at 37ºC with two concentrations of Potassium Bromate: (2.0 mM or 10.0 mM) or vehicle (1 x PBS)
- COMET assay was performed as described in **Table 1**
- Step 7: 2.0 mM and 10.0 mM Potassium Bromate treated cells were incubated for 45 min at 37ºC with FPG (1:5,000) or ENDO III (1:10,000), respectively

Determination of cell viability

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

Data analysis

- ~100 cells per slide were assessed. DNA damage was determined using COMET Assay IV image analysis software and tail intensity (%) was recorded
- Mean and standard deviation (SD) of tail intensities were calculated

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RESULTS

Alkaline COMET assay

Basal DNA damage: Vivotecnia vs published methods[1-5]

- When data generated using Vivotecnia's method was compared to data generated by published methods[1-5], a 50% reduction in the level of basal DNA damage was observed **(Fig. 1)**

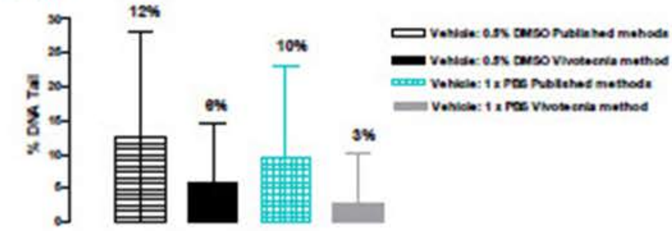

Figure 1: Basal DNA damage levels obtained using Vivotecnia & published methods[1-5]

Etoposide, Methyl methanesulfonate (MMS) and Hydrogen peroxide (H_2O_2)

- Cell viability was >95% after 250 µM Etoposide, 750 µM MMS or 400 µM H_2O_2 treatment
- 250 µM Etoposide, 750 µM MMS and 400 µM H_2O_2 induced 55%, 42% or 72% DNA damage respectively **(Fig. 2)**
- Etoposide treatment resulted in a variable response, the SD was 29.4% **(Fig. 2)**

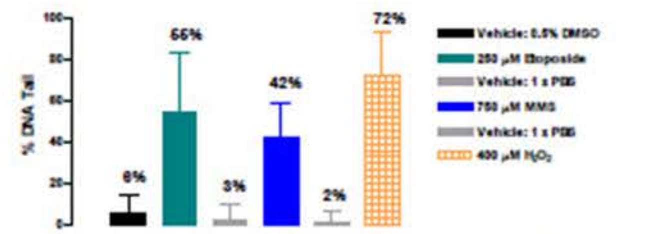

Figure 2. DNA damage in H292 cells after 250 µM Etoposide, 750 µM MMS or 400 µM H_2O_2 treatment

Modified Alkaline COMET assay

Potassium Bromate ($KBrO_3$)

- Cell viability after 2.0 mM and 10.0 mM $KBrO_3$ treatment was >98%
- Minimal DNA damage was induced by 2.0 mM or 10.0 mM $KBrO_3$ <9%
- 2.0 mM $KBrO_3$ + FPG and 10.0 mM $KBrO_3$ + ENDO III induced 71% and 64% oxidative DNA damage respectively **(Fig. 3)**

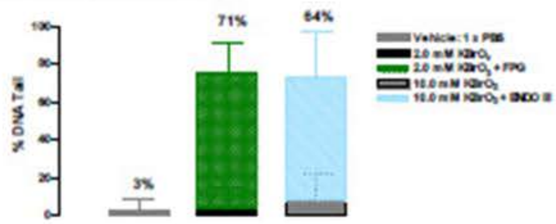

Figure 3. Strand breaks and oxidative DNA damage in H292 cells following incubation with 2.0 or 10.0 mM $KBrO_3$ and subsequent incubation with FPG or ENDO III

CONCLUSIONS

1. **We have successfully developed reproducible methods for:**
   - **the Alkaline COMET assay with MMS and Etoposide**
   - **the Modified COMET assay to show oxidative purine and pyrimidine lesions using $KBrO_3$ with the repair enzymes FPG and ENDO III**
2. **When data generated using our method is compared to data obtained by published methods[1-5], we observe a 50% reduction in basal DNA damage**

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