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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 compounds¹⁻²
- We have established optimal methods to determine DNA damage by:
 - The Alkaline COMET assay which detects DNA strand breaks, alkali-labile sites (ALS) and DNA-DNA/DNA-protein cross linking
 - 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 performed¹⁻⁵ (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

* 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 1.2×10^6 cells/well

Alkaline COMET assay

- Confluent H292 cells were incubated for 1 hour at 37°C with Etoposide (250 µM), Methyl methanesulfonate (MMS; 750 µM), Hydrogen peroxide (400 µM; H₂O₂) 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 37°C 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 37°C 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 methods¹⁻⁵

- When data generated using Vivotecnia's method was compared to data generated by published methods¹⁻⁵, a 50% reduction in the level of basal DNA damage was observed (Fig. 1)

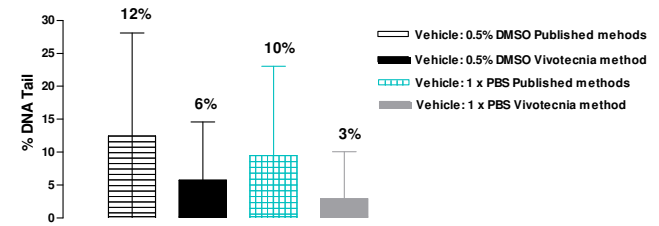


Figure 1: Basal DNA damage levels obtained using Vivotecnia & published methods¹⁻⁵

Etoposide, Methyl methanesulfonate (MMS) and Hydrogen peroxide (H₂O₂)

- Cell viability was >95% after 250 µM Etoposide, 750 µM MMS or 400 µM H₂O₂ treatment
- 250 µM Etoposide, 750 µM MMS and 400 µM H₂O₂ induced 55%, 42% or 72% DNA damage respectively (Fig. 2)
- Etoposide treatment resulted in a variable response, the SD was 29.4% (Fig. 2)

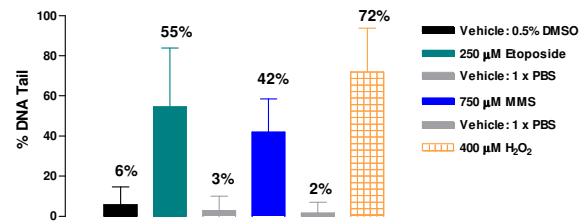


Figure 2: DNA damage in H292 cells after 250 µM Etoposide, 750 µM MMS or 400 µM H₂O₂ treatment

Modified Alkaline COMET assay

Potassium Bromate (KBrO₃)

- Cell viability after 2.0 mM and 10.0 mM KBrO₃ treatment was >98%
- Minimal DNA damage was induced by 2.0 mM or 10.0 mM KBrO₃ <9%
- 2.0 mM KBrO₃ + FPG and 10.0 mM KBrO₃ + ENDO III induced 71% and 64% oxidative DNA damage respectively (Fig. 3)

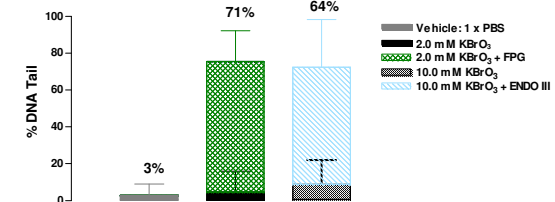


Figure 3: Strand breaks and oxidative DNA damage in H292 cells following incubation with 2.0 or 10.0 mM KBrO₃ and subsequent incubation with FPG or ENDO III

CONCLUSIONS

- 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 KBrO₃ with the repair enzymes FPG and ENDO III
- When data generated using our method is compared to data obtained by published methods¹⁻⁵, we observe a 50% reduction in basal DNA damage

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