INTRODUCTION

- The single-cell gel electrophoresis assay (COMET assay) is a sensitive and rapid method to measure DNA damage and identify potential genotoxic compounds.
- 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 (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)

RESULTS

- Alkaline COMET assay
  - Basal DNA damage: Vivotecnia vs published methods
  - When data generated using Vivotecnia’s method was compared to data generated by published methods, a 50% reduction in the level of basal DNA damage was observed (Fig. 1)

- Modified Alkaline COMET assay
  - 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)

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

REFERENCES

2. Vivotecnia method for the COMET assay
   - NCI-H292 cells were supplied by ATCC (http://www.ics standards-atcc.org)
   - The day before COMET analysis, cells were seeded in 6-well plates at 1.2x10^4 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

Table 1: Stages investigated to optimise COMET methods

<table>
<thead>
<tr>
<th>Step</th>
<th>Published methods</th>
<th>Vivotecnia method</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Slide preparation</td>
<td>Normal slides</td>
<td>Superfrost slides</td>
<td>To avoid agarose detachment during Electrophoresis - step 8</td>
</tr>
<tr>
<td>1.3</td>
<td>1.0% agarose</td>
<td>1.5% agarose</td>
<td>N/A</td>
</tr>
<tr>
<td>2. Cell culture</td>
<td>24 well plates</td>
<td>6-well plates (high number of cells/well)</td>
<td>Sufficient cells for automatic counting</td>
</tr>
<tr>
<td>2.2</td>
<td>Neutrophil chamber</td>
<td>Automatic cell counter</td>
<td>Decrease the time to Lysis – step 4</td>
</tr>
<tr>
<td>2.3</td>
<td>Neutrophil chamber</td>
<td>Third agarose layer</td>
<td>Decrease the time to Lysis – step 4</td>
</tr>
<tr>
<td>2.5</td>
<td>5-7 min cooling</td>
<td>2 min using pre-cooled metallic plate</td>
<td>Reduce agarose melting</td>
</tr>
<tr>
<td>3. Lysis</td>
<td>~1 hour</td>
<td>~24 hours</td>
<td>Ensure full cellular lysis</td>
</tr>
<tr>
<td>4. Wash</td>
<td></td>
<td></td>
<td>Improve removal of detergents and salts</td>
</tr>
<tr>
<td>5. Alkaline unwinding</td>
<td>pH ~13</td>
<td>pH ~13</td>
<td>Maximize detection of DNA alkali-labile sites and strand breaks</td>
</tr>
<tr>
<td>6. Enzyme incubation*</td>
<td>45 min</td>
<td>45 min</td>
<td>N/A</td>
</tr>
<tr>
<td>7. Electrophoresis</td>
<td>Conventional electrophoresis tank</td>
<td>COMET electrophoresis tank (Thistle Scientific)</td>
<td>Reduce levels of basal DNA damage and protect against UV degradation</td>
</tr>
<tr>
<td>9. Neutralisation</td>
<td>~1.5 mL Neutralisation buffer/slide</td>
<td>~3.5 mL Neutralisation buffer/slide</td>
<td>Effective removal of Electrophoresis buffer</td>
</tr>
<tr>
<td>10. Fixation</td>
<td>100% ethanol</td>
<td>Air-dry for ~24h</td>
<td>Improve staining</td>
</tr>
<tr>
<td>11. COMET visualisation</td>
<td>Ethidium bromide</td>
<td>DAPI</td>
<td>Avoid use of teratogenic compounds</td>
</tr>
</tbody>
</table>

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

Figure 1: Basal DNA damage levels obtained using Vivotecnia & published methods

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

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