Evaluation of manual and image analysis quantification of DNA damage in the alkaline comet assay

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The alkaline comet assay or single cell microgel electrophoresis assay is a sensitive method of detecting DNA strand breaks and alkali labile sites in individual cells. The results of this assay can be analysed by different methods. In this study we compared analyses of the same slides by a manual method and by image analysis, post-treatment of clone 707 Friend erythroleukaemia cells with H$_2$O$_2$. The parameters which were found to be particularly useful were: comet length and comet area (measured manually) and percentage tail DNA, tail moment, tail length and tail length/head radius (L/H), measured using image analysis. The manual method for comet analysis presented in this paper would appear to provide good and reliable comet data. However, the image analysis comet system described offers an alternative analysis method which avoids the need for photomicrographs and tedious manual analysis. The image analysis parameters: % tail DNA, tail moment, tail length and L/H give good consistent results and for large-scale analysis it will, therefore, conceivably be the method of choice.

Introduction

The alkaline comet assay or single cell microgel electrophoresis assay (SCGE) (Singh et al., 1988) is a method of detecting DNA strand breaks and alkali labile sites in individual cells. Since its introduction in 1988, it has become accepted as a rapid, simple and sensitive visual technique for measuring DNA breakage which has found application in DNA damage and repair studies, biomonitoring, analysis of irradiated food and repair studies, biomonitoring, analysis of irradiated food and repair studies, biomonitoring, analysis of irradiated food.

Materials and methods

Culture of 707 cells

Clone mouse FEL cells which were isolated by Scher et al. (1971) were grown in Ham’s SF12 medium, supplemented with 16% horse serum, L-glutamine and antibiotics (penicillin and streptomycin). Population doubling times were 12 h.

Cultures were maintained at cell concentrations between 1x10$^4$ and 8x10$^4$ cells/ml. Cell concentrations were determined using an electronic particle counter (Coulter Counter model DN). The cells were routinely grown in 75 cm$^2$ plastic flasks with vented lids at 37°C in a 5% CO$_2$-in-air humidified atmosphere.

Treatment of cells

H$_2$O$_2$ was used to introduce hydroxyl radical-induced DNA damage. The cells were treated at a concentration of 5x10$^4$ cells/ml with 5, 10 and 15 μM H$_2$O$_2$ in SF12 growth medium at 4°C for 30 min. The cells were then washed with phosphate-buffered saline (PBS) and for repair studies were resuspended in fresh growth medium and then incubated at 37°C for appropriate repair times.

Preparation of slides

The preparation of slides was carried out under yellow light to prevent any additional DNA damage. Darlin fully frosted microscope slides were each covered with 100 μl of 0.5% normal melting point agarose in Ca$^{2+}$- and
Mg$^{2+}$-free PBS at 45°C. They were immediately covered with a large no. 1 coverslip and then kept at 4°C until the agarose had solidified.

Cells for analysis were pelleted at 2500 r.p.m at 4°C for 4 min, resuspended in 10 µl of PBS and then mixed with 75 µl of 0.5% low melting point agarose (LMA) at 37°C. After gently removing the coverslip the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip and allowed to solidify at 4°C. The slides (without coverslips) were immersed in freshly prepared, cold lysing solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris pH 10, with 1% Triton X-100 added just before use) (Singh et al., 1988) for a minimum of 1 h and a maximum of 4 days, at 4°C. No effect is observed when using different lysing times between 1 h and 4 days.

Slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side with the agarose end facing the anode. Electrophoresis buffer was prepared by mixing a stock solution of 600 mM NaOH with 200 mM Na$_2$EDTA in a 6:1 ratio, and diluting with H$_2$O. The results in a working solution of 300 mM NaOH and 1 mM Na$_2$EDTA at pH 13.5. The tank was filled with fresh electrophoresis buffer at 12-15°C to a level of 25 cm above the slides. The electrophoresis tank was placed on a tray of ice to maintain the temperature below 15°C. The slides were left in the high pH buffer for 20 min to allow unwinding of the DNA to occur before electrophoresis.

Electrophoresis was conducted for 20 min at 25 V (0.66 V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and flooded slowly with three changes of neutralization buffer (0.4 M Tris pH 7.5) for 5 min each. This removes alkali and detergents which would interfere with ethidium bromide staining. The slides were again drained before being stained with 50 µl of 20 µg/ml ethidium bromide and a coverslip was placed on top.

**Comet capture and analysis**

Slides were viewed up to 24 h after staining. No difference was observed between slides viewed immediately after staining and slides viewed up to 24 h after staining. Analysis was performed using a x40 objective with a Nikon epiphotaphase microscope equipped with an excitation filter of 515-560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. A total of 50 individual comets were analysed for each population investigated. Cells were selected by moving from top to bottom along the slide to avoid analysis of the same comet twice. Selection was random with no attempt to select particular comets other than to avoid obvious debris and any comets that lay too close together for accurate image resolution. The same slides were used for both the manual evaluation and image analysis. Image analysis of slides was performed first as it involves least exposure to UV light, and hence less bleaching of images, due to the speed of the assay procedure.

**Manual measurement**

Photomicrographs were taken at X400 magnification using Kodak T-max 400 ASA black and white 35 mm film. The head diameter and comet length (i.e. head + tail length) were measured, using Mitutoyo 0–150 mm RS digital callipers, by placing the negative over a light box. The area of the comet was measured by placing a mm$^2$ graph transparency between the light box and the negative and then counting the number of squares which the comet covered.

**Image analysis measurements**

Image analysis of comets was achieved using the Komet version 3.0 assay system previously mentioned (Kinetic Imaging Ltd). This system is based on the comet parameter definitions for computer based analysis first used by Olive et al. (1990). The digital comet assay system provides a wide range of quantitative parameters to describe the densitometric and geometric properties of the comet. This includes skewness, kurtosis, mean, mode, intensity, % head DNA, % tail DNA, tail length, comet area, head extent, tail length/head radius (L/H) and tail moment. Data for the latter six parameters only is presented here. The position and intensity of the comet head is determined by assuming that the head region is symmetrical and all pixels in excess of the calculated area of the head are defined to be part of the comet tail. The tail length measure is defined as the distance from the centre of the head to the end of the tail, head extent = (right edge - left edge)×image X-resolution where right and left edge are found by intensity searching and controlled by head threshold. The percentage tail DNA is the relative total intensity of DNA in the tail and tail moment is the % DNA in the tail×the distance between the head and tail centre/100. Results are stored on a spread sheet using the Microsoft Excel package.

**Results**

It was expected that the FEL cells should show dose dependent increases in DNA damage immediately following H$_2$O$_2$ treatments (T = 0). It was also envisaged that after 60 min repair the amount of DNA damage remaining would be considerably reduced. The precise kinetics of H$_2$O$_2$ repair in FEL cells is not known, but it is also expected that some DNA repair will have been completed following 30 min repair incubation. Optimal measures were considered to be those which gave the expected trends and dose responses, and which showed consistency between experiments.

The results obtained for each of the manual measurements, head diameter, comet length, comet length/head diameter (CL/HD), and area when FEL cells were treated with H$_2$O$_2$ are shown in Figure 2a-d. The expected trends, with a dose-dependent increase in DNA damage were observed with comet length, CL/HD and area at T = 0, and a consistent decrease in damage following 60 min repair time at each dose. Reduced DNA damage for the two higher doses of H$_2$O$_2$ used, is also observed at 30 min repair times using the above three manual parameters. The image analysis data is presented in Figure 3a–f, and it can be seen that for % tail DNA, tail length, head extent/2, tail moment, L/H and area dose-dependent increases were observed immediately following H$_2$O$_2$ treatments (T = 0). It should be noted that head extent/2 actually increases with H$_2$O$_2$ dose where one might expect it to decrease with increasing damage. This may be explained by the ‘halo’ effect, where, with increasing strand breaks, the DNA has become less supercoiled in the nuclear region during the unwinding stage and so the size of the head extent/2 has increased. After 60 min repair following H$_2$O$_2$ treatment the DNA damage
Fig. 2. DNA damage processing as measured by the comet assay, on FEL cells treated with 5, 10 and 15 μM H2O2, measured manually using (a) head diameter (mm); (b) comet length (mm); (c) CL/HD (comet length/head diameter); and (d) area (mm²). Each value represents the mean ± SE of two independent experiments, with 50 cells analysed in each experiment.

remaining, as measured by each of the parameters a–f, was observed to be reduced at each dose studied. The decreased head extent/2 values obtained at 60 min with each dose of H2O2 examined are consistent with the reduction of the halo effect, giving rise to decreased head diameter with increased repair. The results using head extent/2 do not, however, show the expected trends as clearly as do the other parameters. This may be due to the physically small measurements involved relative to the other measures. Consistent decreases in DNA damage following 30 min repair relative to T = 0 min following 10 and 15 μM H2O2 are observed using % tail DNA, tail length, L/H and tail moment. Area measures even at 10 μM H2O2 following 30 min repair do not show the expected decrease in DNA damage. Following 30 min repair after 5 μM H2O2 all six image analysis parameters were elevated relative to T = 0 min. It is clear from Figures 2 and 3 that there are significant differences between head diameter and head extent measures as obtained manually and by image analysis respectively. This may be due to the way in which the two measurements are obtained. In manual head diameter the diameter is measured perpendicular to the direction of the tail, while in image analysis the measures are made from the left and right edges of the head extent. If the head is not spherical, differences will be apparent in the two measurements obtained. The manual head diameter parameter does not appear to provide any useful data.

The distribution of DNA damage among cells within a dose of H2O2 is shown in Figure 4 and was observed to be similar with both image analysis (tail length) and manual (comet length) methods of analysis. Both methods of analysis resulted in a normal distribution of damage. It can also be seen from Figure 4 that the variability between experiments was also similar, for both methods of analysis. Similar results were obtained for the other doses used (data not shown).

Discussion

From the results presented in Figure 2 it is clear that the manual method for analysis gives good reliable results and
three of the parameters are in agreement: comet length, CL/HD and area all appear to be suitable for analysis. The manual head diameter values obtained did not display any clear trends and this measure was therefore not considered to be a useful parameter.

From the results presented in Figure 3, it can be seen that the image analysis parameters which best demonstrate the expected trends are % tail DNA, tail moment, tail length and L/H. It should be noted that Olive et al. (1992) have shown that when electrophoresis is carried out at low voltage, e.g. in the neutral assay or in a modified alkali method the observable tail length, except at low doses is largely independent of radiation dose. Under these conditions % tail DNA was the best measure of DNA damage. For consistency between the
Quantification of DNA damage in the alkaline comet assay

Fig. 4. Distribution of damage following (a) 15 μM and (b) 10 μM H₂O₂ as measured by (a) manual comet length and (b) image analysis tail length. Each histogram represents the mean ± SE of two independent experiments with 50 cells analysed in each experiment.

Table I. Breakdown of time required to analyse 50 cells by (A) manual and (B) image analysis

<table>
<thead>
<tr>
<th>A</th>
<th>Comet length</th>
<th>Head diameter</th>
<th>Comet area</th>
<th>All three parameters</th>
</tr>
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<tbody>
<tr>
<td>Manual measurements</td>
<td></td>
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<tr>
<td>(50 cells)</td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>Taking photomicrographs (min)</td>
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<tr>
<td>Developing films (min)</td>
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<td>40</td>
<td>40</td>
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<tr>
<td>Measuring parameters (min)</td>
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<td>18</td>
<td>34</td>
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<tr>
<td>Results in spreadsheet format (min)</td>
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<td>3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Total time required for manual analysis (min)</td>
<td>96</td>
<td>96</td>
<td>106</td>
<td>128</td>
</tr>
</tbody>
</table>

| B                        |              |               |            |                      |
| Total time required for all measurements in spreadsheet format (min) | 15 |

various assays the tail length measure may therefore be best avoided or used in combination with another measure not relying solely on tail length when extensive DNA damage is to be evaluated. % tail DNA, tail moment, tail length and L/
HD) at T = 30 min relative to T = 0 min following 5 μM H₂O₂ treatment may be due to the nature of the repair process itself and the type of lesions incurred in the DNA. DNA strand breaks are formed when cells are exposed to H₂O₂, in addition several types of hydroxyl radical induced base damage have been detected by gas chromatography–mass spectroscopy analysis, (Dizdaroglu et al., 1991). In order to remove a lesion from the DNA a strand break may be introduced which will be interpreted as an increase in damage in this assay. Hence, an increase in damage detected at T = 30 may reflect a stage of the repair process where particular lesions are being removed.

Overall it would appear, therefore, that the manual method offers a good and reliable method of analysing comet slides. However, it is considerably slower than the image analysis method. Using the manual method it can take ~2 h in comparison with 15 min with the image analysis system to analyse 50 images (for breakdown of the times involved in our analysis see Table I). Manual measurements made directly through the eyepiece of a microscope provide a quicker method than either the manual analysis method evaluated in this paper or image analysis. However, the eyepiece manual method cannot provide any information regarding the area of the comet, nor any densitometric information on the fluorescence observed. Since increased tail fluorescence is not always accompanied by increased tail length or indeed increased comet area, image analysis provides a superior analysis in that densitometric measures are obtained.

In conclusion, the manual method for comet analysis presented in this paper would appear to provide good and reliable comet data. The image analysis comet system described here offers an alternative method for analysis avoiding the need for photomicrographs and tedious manual analysis. The image analysis parameters, especially % tail DNA, tail moment, tail length and L/H give good consistent results which display the expected trends and are in agreement with results obtained from manual analysis. Both manual and image analysis methods demonstrate consistency between experiments, however, due to the ability of image analysis to quantify fluorescence intensity and its rapid delivery of results, for large scale analysis it will therefore conceivably be the method of choice.

Acknowledgement

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References


