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The influence of the number of cells scored on the sensitivity in the comet assay

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ABSTRACT

The impact on the sensitivity of the *in vitro* comet assay by increasing the number of cells scored has only been addressed in a few studies. The present study investigated whether the sensitivity of the assay could be improved by scoring more than 100 cells. Two cell lines and three different chemicals were used: Caco-2 cells were exposed to ethylmethane sulfonate and hydrogen peroxide in three concentrations, and HepG2 cells were exposed to ethylmethane sulfonate, hydrogen peroxide and benzo[*a*]pyrene in up to four concentrations, in four to five independent experiments. The scoring was carried out by means of a fully automated scoring system and the results were analyzed by evaluating the % tail DNA of 100–700 randomly selected cells for each slide consisting of two gels. By increasing the number of cells scored, the coefficients of variance decreased, leading to an improved sensitivity of the assay. A two-way ANOVA analysis of variance showed that the contribution from the two variables "the number of cells scored" and "concentration" on the total variation in the coefficients of variance dataset was statistically significant ($p < 0.05$). The increase in sensitivity was demonstrated by the possibility to detect an increase in % tail DNA with statistical significance at lower concentrations. The results indicated that for low levels of DNA damage, below 9% tail DNA, scoring of 600 cells increased the sensitivity compared with scoring of 100 cells. For relatively low levels of DNA damage, about 9–16% tail DNA, scoring of 300 cells increased the sensitivity. Thus, the recommendation for the optimum number of cells scored would be 600 and 300 for low and relatively low levels of DNA damage, respectively. The findings from this study could be particularly important for bio-monitoring studies where small differences in DNA-damage levels could be relevant.

1. Introduction

The comet assay or single-cell gel electrophoresis is a well-established genotoxicity test that is becoming increasingly popular for the detection of a broad spectrum of DNA damage with high sensitivity. It is now widely used in regulatory, mechanistic and bio-monitoring studies. The assay is very useful in *in vitro* studies but also in *in vivo* studies, where appropriate target organ/organs can be analyzed for the presence of DNA strand-breaks. For the *in vitro* comet assay an internationally accepted working guideline has been published [1]. One of the potential advantages is that the assay can potentially be used as a high-throughput screening assay. The *in vitro* version of the assay has been widely used in bio-monitoring studies over the past twenty years [2]. An improvement in sensitivity of the assay could provide enhanced reliability in bio-monitoring studies. Several parameters in the comet assay have been investigated, such as agarose concentration, unwinding time and electrophoresis conditions [3,4], and optimization of these parameters was shown to improve assay sensitivity. Increasing the number of cells scored may be another method to improve the sensitivity. At present, there is no consensus about the recommended number of cells scored and usually data on 100 cells per sample are reported for *in vitro* studies. Most laboratories today use semi-automated scoring techniques with integrated software. The selection of the cells to be scored is performed manually: one cell is selected and with a single click (sometimes more clicks) with the PC mouse the damage is quantified, and then the next cell is selected and so on. Scoring more than 100 cells per sample would be too time-consuming. Fully-automated scoring systems provide the possibility for faster scoring of more than 100 cells per sample; furthermore, it allows scoring a more random selection of cells compared with semi-automated scoring techniques. Several studies have reported the use of automated scoring, both with in-house systems and with commercially available equipment [5–9]. However, the impact on the sensitivity of the assay by increasing the number of cells scored has not yet been fully investigated and only a few studies have addressed the issue [6,10,11]. The aim of this study was to investigate if the assay sensitivity could be improved by increasing the number of cells scored in independent samples,

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compared with the standard number of 100 cells per sample. The study explored the possibility to detect significant differences at relatively low levels of DNA damage.

2. Materials and methods

2.1. Cells and test compounds

Caco-2 cells (human colon cancer cells) were kindly donated by the Department of Microbiology, National Food Institute, Technical University of Denmark. The cells were grown in DMEM (Gibco No. 11039) supplemented with 10% foetal bovine serum (FBS) and 10 ml penicillin (10000 Units/ml) and streptomycin (10000 μg/ml) per liter (Gibco No. 15148-114). Cells were used at passage numbers between 4 and 14. HepG2 cells (human hepatocellular carcinoma cells) were kindly donated by Dr Martin Roursgaard, Institute of Public Health, University of Copenhagen. The cells were grown in Advanced MEM (GIBCO No. 12492) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine per liter. Cells were used at passage numbers between 5 and 13. One ml of Caco-2 cells at 2×10^6 cells/ml was treated with 0, 96, 190 and 370 μM ethylmethane sulfonate (EMS) and 10, 20 and 30 μM of hydrogen peroxide (H_2O_2). One ml of HepG2 cells at 2×10^6 cells/ml was treated with 0, 0.2, 0.4 and 0.8 μM of benzo[*a*]pyrene (B[*a*]P), 0, 100, 200, 400 and 1000 μM of EMS, or with 5, 10, 20 and 30 μM of H_2O_2. The cell cultures were treated for 2 h at 37 °C (EMS and B[*a*]P) or for 5 min at 4 °C (H_2O_2). B[*a*]P was dissolved in DMSO (final concentration, 2%). We tested the influence on cell viability (Nucleocounter, NC 3000, Chemometec, Allerød, Denmark) of DMSO concentrations up to 10%: cell viability was affected at 4% DMSO and above. Therefore, 2% DMSO was chosen. EMS and H_2O_2 were dissolved in cell-culture medium. After the treatment, cells were centrifuged, washed with fresh medium and phosphate-buffered saline (PBS), and suspended at 2000–4000 cells/ml in PBS, after which the cell viability was determined (Nucleocounter, NC 3000, Chemometec, Allerød, Denmark). The HepG2 cell line was used because it is very suitable for genotoxicity testing, which has been investigated in a comprehensive review [12]. The Caco-2 cell line is a model of the intestinal barrier, which is very useful for examining effects of toxic agents after oral exposure, and very relevant in food toxicology [13]. EMS and H_2O_2 were chosen because they are genotoxic and are often used as positive controls in the comet assay. B[*a*]P was used because it has been tested as a weakly genotoxic to non-genotoxic compound in the comet assay with HepG2 cells [14,15]. Except for B[*a*]P, the concentrations were chosen in order to obtain a concentration-response effect. Cytotoxic concentrations were avoided. None of the concentrations tested were cytotoxic, the only exception was 0.8 μM of benzo[*a*]pyrene, which showed signs of low cytotoxicity towards HepG2 cells, with about 15% lower cell viability compared with control samples.

2.2. Comet assay

The slides for the comet assay were prepared according to Tice et al. [1] as described previously [16], with some minor modifications according to the manufacturer of the CometAssay® Kit (Trevigen, Gaithersburg, MD, USA). Briefly, suspensions containing 2000–4000 cells were mixed with 150 μl of molten low melting-point agarose. Fifty μl of the mixture was applied onto each of the two gels of a Trevigen slide. After lysis, electrophoresis, neutralization and fixation, the DNA was stained with 10 μl SYBR Green on both gels of the slides and a drop of anti-fading solution (500 mg *p*-phenylenediamine dihydrochloride in 4.5 ml PBS) was added to each gel to avoid fading. After staining, the scoring of slides was carried out within 6 h. For each concentration, four to five slides (samples) were prepared in four to five independent experiments (one slide in each experiment). The comet distributions of the samples showed non-normal distributions, therefore median values of the '% tail DNA' from each sample were used and the means of the median values were calculated.

2.3. Fully-automated and semi-automated scoring systems for the comet assay

Fully-automated comet assay scoring was performed with the Pathfinder™ Cellscan Comet-imaging system (IMSTAR, Paris, France). The complete system was mounted onto a voltage-stabilizing unit to avoid possible disturbances of voltage fluctuation. The system comprises a microscope (Olympus, model BX41TF, Japan) equipped with proprietary motorizations for the *X*-, *Y*- and *Z*-axis and a fluorescence epi-cubes turret, a high-resolution (1360 × 1024 pixels of 6.45 μm × 6.45 μm) 12-bits dynamics digital camera (Jenoptik, ProgRes MF, Germany). The Pathfinder™ Cellscan Comet software module performs a fully-automated image capture including autofocus of all comet images of the sample, detection of comets, *i.e.* the cell's body contour, separating accurately the head and tail, and quantification of morphology and fluorescence-intensity characteristics for each individual comet, thus enabling analysis based on parameter distribution. Image capture was performed at 10× magnification with a green narrow-band FITC filter and a metal halide fluorescence lamp, with a capacity of four slides per batch.

After the slides were loaded in the holder, five focus positions or Z-landmarks for automatic focusing were set; the automatic focus is performed on positions within the two scoring zones (corresponding to two gels) to be scanned and subsequent focus positions were calculated based on the predefined focus positions. Four positions were set in each corner in a rectangular pattern of the scoring zones and one position in the middle. Hereafter, the zones were scanned and all corresponding images captured. In the present study, the time for capturing images on one slide was about 5 min. After one day of slide capturing, the comets were automatically quantified overnight with the Pathfinder™ Batch Processor software, which allows parallelization of large image-analysis algorithms by splitting the image in sub-images that are automatically exported as a text file for calculations.

The automated detection of comets is based on advanced mathematical morphology techniques used in an optimized algorithm specifically adapted to comet cell type, shape, and to damage dynamics. The different steps are summarized below:

(1) Identification of comet candidates, after segmentation by threshold of objects from background in predefined scanning zones. Background is automatically analyzed and corrected for, so that this noise component has a flat baseline, thus ensuring that the light is homogeneous within each comet. (2) Junction of individual particles belonging to the same comet. (3) Filtering of detected objects not corresponding to comets, using bracketed values of characteristics in terms of convexity, area size, fluorescence intensity and symmetry. (4) Head detection by criteria of brighter fraction and recognition of head center. (5) Migration's direction filtering to eliminate remaining artifacts. The total number of detected comets only depends on the density of cells on the slide. Several quantification parameters are available (% tail DNA), Olive-tail moment, comet-tail moment, tail length as well as the parameters of mean and median values. In the present study, % tail DNA and median values of the comets scored on each slide were used. For semi-automated scoring, two different software products were used. The setup consisted of a Leica DMR fluorescence microscope (40× objective) coupled *via* an 8-bits dynamics CCD camera to a Kinetic Imaging 5.5 (UK) or Perceptive IV image-analysis system. In our laboratory we updated our semi-automated scoring system during the experiments, and therefore the scoring was performed with two different systems. With semi-automated scoring, one cell was selected manually, the damage quantified by the software, then the next cell was selected and so on. Fifty cells were scored on both gels on each slide, giving 100 cells per slide (sample). Data from the fully-automated system were exported to Excel. The numbers of cells scored on the slides were dependent on the cell density. In the present study, usually 500–1000 cells were present on each gel and this cell density was acceptable. The frequency of overlapping cells was low. Up to 700 cells (350 cells on each gel) were evaluated because this was the minimum number of cells present on each gel. The randomization of 50–350 cells per gel (100–700 cells for each slide) was done by macro-programming in Excel so that cells across the entire gel were evaluated. The same operator scored all the slides measured with the semi-automated systems. With the fully-automated system there is no operator variability, which was verified before the study. The same operator worked with all the slides that were analyzed with the fully-automated system.

2.4. Statistics

Differences in % tail DNA between the samples from treated and control groups (Table 1) were analyzed by one-way ANOVA, with Dunnet's test to compare the three concentrations *vs* the corresponding control group. Investigation of the influence of the concentrations and the number of cells scored on the coefficients of variance (CVs) in Table 3 was done by a two-way ANOVA. There was no interaction between number of cells scored and concentrations of chemical agents. Regression coefficients (R^2 values) of the different experiments and for the different numbers of cells scored were calculated by linear regression analysis, in which all the single data for each concentration were used (Table 1). SAS Enterprise 3.0 and GraphPad Prism 5.0 were used as statistical software.

3. Results

Table 1 shows the % tail DNA of 100–700 cells scored by the fully-automated system. For Caco-2 cells exposed to either EMS or H_2O_2, there was a clear effect of the number of cells scored with respect to assay sensitivity, and the scoring of 300 cells or more resulted in a statistically significant increase in % tail DNA compared with controls, at all three concentrations. When scoring 200 cells or less, a statistically significant increase in % tail DNA was only obtained at the medium and/or the highest concentrations. The results further show that it was possible to increase the assay sensitivity for samples with 9–11% tail DNA, which is considered to represent a relatively low level of DNA damage.

A similar relationship between the number of cells scored and sensitivity of the assay was seen for HepG2 cells treated with B[*a*]P, EMS, or H_2O_2. More specifically, when scoring 300 or more cells treated with EMS, a statistically significant increase in % tail DNA was observed at all four concentrations compared with control

Table 1
Results of % tail DNA (mean ± S.D. of median values of independent samples). Caco-2 and EMS, $n = 5$. Caco-2 and H_2O_2, $n = 4$. HepG2 and B[*a*]P, $n = 5$. HepG2 and EMS, $n = 5$. HepG2 and H_2O_2, $n = 4$. One way ANOVA analysis of variance with Dunnett's test to compare the concentrations versus the corresponding control group was used. Values in bold are statistically significantly different from the corresponding controls. With the semi-automatic systems 100 cells were scored. With the fully-automatic system data analysis was carried out by scoring 100–700 randomly selected cells. Regression coefficients for linear regression were calculated.

EMS (μM), Caco-2 cells	0	96	190	370	Regression coefficients R^2 values
100 cells (SA)$^{\infty}$	3.6 ± 1.6	9.4 ± 7.6	10.3 ± 2.3	**17.4 ± 9.4****	0.42
100 cells (FA)	1.7 ± 1.3	8.2 ± 6.7	9.5 ± 5.1	**21.3 ± 7.5*****	0.64
200 cells	1.5 ± 1.2	8.9 ± 7.4	**9.7 ± 4.8***	**21.7 ± 6.4*****	0.66
300 cells	1.6 ± 1.2	**9.0 ± 6.9***	**8.9 ± 4.4***	**22.4 ± 6.2*****	0.69
400 cells	1.6 ± 1.1	**9.1 ± 6.8***	**10.0 ± 4.5***	**22.9 ± 5.9*****	0.71
500 cells	1.6 ± 1.1	**8.4 ± 5.4***	**9.9 ± 4.0***	**22.7 ± 6.0*****	0.75
600 cells	1.5 ± 0.9	**8.3 ± 5.3***	**10.0 ± 3.9****	**22.9 ± 5.7*****	0.77
700 cells	1.5 ± 1.0	**8.4 ± 5.5***	**10.6 ± 4.2****	**23.4 ± 5.5*****	0.78
H_2O_2 (μM), Caco-2 cells	0	10	20	30	Regression coefficients R^2 values
100 cells (FA)	2.7 ± 0.6	10.7 ± 3.3	**22.0 ± 10.1****	**38.9 ± 9.0*****	0.81
200 cells	2.3 ± 1.0	11.1 ± 3.5	**23.0 ± 9.2*****	**40.4 ± 6.6*****	0.86
300 cells	2.4 ± 1.0	**11.7 ± 3.5***	**22.6 ± 8.8*****	**41.9 ± 4.9*****	0.88
400 cells	2.6 ± 0.3	**11.2 ± 2.7***	**22.4 ± 8.0*****	**42.5 ± 4.0*****	0.90
500 cells	2.6 ± 0.3	**11.2 ± 3.0***	**23.2 ± 6.7*****	**43.0 ± 4.4*****	0.91
600 cells	2.6 ± 0.2	**10.9 ± 3.8***	**22.8 ± 6.7*****	**43.1 ± 3.8*****	0.91
700 cells	2.5 ± 0.2	**11.5 ± 1.9***	**21.8 ± 5.6*****	**42.4 ± 3.8*****	0.92
B[*a*]P (μM), Hep-G2 cells	0	0.2	0.4	0.8	Regression coefficients R^2 values
100 cells (SA)$^{\in}$	4.4 ± 2.9	6.1 ± 3.2	6.6 ± 2.3	7.5 ± 2.8	0.15
100 cells (FA)	2.8 ± 1.9	4.9 ± 2.8	5.4 ± 3.0	6.1 ± 2.0	0.20
200 cells	2.7 ± 1.8	4.4 ± 3.0	5.2 ± 3.1	5.5 ± 1.7	0.14
300 cells	2.8 ± 1.6	5.0 ± 3.6	5.3 ± 3.1	5.8 ± 2.1	0.12
400 cells	3.0 ± 1.7	4.9 ± 3.0	5.3 ± 2.5	5.8 ± 2.1	0.15
500 cells	2.9 ± 1.5	5.1 ± 2.9	5.2 ± 2.5	**6.0 ± 1.7***	0.19
600 cells	3.0 ± 1.5	5.0 ± 2.5	5.7 ± 2.5	**6.4 ± 1.3***	0.28
700 cells	3.0 ± 1.3	4.9 ± 2.3	5.6 ± 2.3	**6.5 ± 1.2****	0.33
EMS (μM), Hep-G2 cells$^{\beta}$	0	100	200	400	1000	Regression coefficients R^2 values
100 cells (FA)	2.6 ± 2.8	12.2 ± 9.9	14.2 ± 9.1	15.7 ± 11.0	**40.2 ± 34.0****	0.38
200 cells	2.3 ± 2.2	12.7 ± 9.4	**14.0 ± 9.3***	**16.4 ± 11.7***	**34.8 ± 26.2*****	0.38
300 cells	2.3 ± 2.1	**12.0 ± 8.4***	**13.4 ± 8.9***	**15.8 ± 10.3***	**33.0 ± 24.0*****	0.40
400 cells	2.2 ± 1.6	**12.6 ± 8.0***	**13.7 ± 7.8***	**17.1 ± 11.0****	**36.0 ± 25.3*****	0.44
500 cells	2.3 ± 1.6	**12.2 ± 7.7***	**13.6 ± 8.2***	**16.7 ± 10.8****	**41.3 ± 28.6*****	0.48
600 cells	2.3 ± 1.5	**11.8 ± 7.1***	**13.8 ± 8.5***	**17.2 ± 10.7****	**41.1 ± 28.2*****	0.49
700 cells	2.3 ± 1.4	**12.3 ± 7.4***	**13.4 ± 7.3***	**17.1 ± 10.4****	**42.0 ± 28.9*****	0.49
H_2O_2 (μM), Hep-G2 cells	0	5	10	20	30	Regression coefficients R^2 values
100 cells (FA)	2.9 ± 1.4	4.9 ± 2.4	4.6 ± 2.7	**7.5 ± 1.2***	**16.7 ± 5.3*****	0.62
200 cells	2.8 ± 1.4	4.7 ± 2.2	5.3 ± 2.8	**7.4 ± 1.4***	**16.1 ± 3.8*****	0.65
300 cells	2.9 ± 1.2	4.7 ± 2.1	5.5 ± 2.3	**7.5 ± 1.0****	**15.2 ± 3.7*****	0.70
400 cells	2.9 ± 1.1	4.8 ± 1.9	5.4 ± 2.2	**7.7 ± 0.6****	**14.9 ± 3.4*****	0.72
500 cells	3.0 ± 1.1	4.7 ± 2.0	5.5 ± 1.9	**7.5 ± 0.9****	**14.8 ± 2.9*****	0.73
600 cells	3.0 ± 1.1	4.6 ± 1.9	**5.7 ± 1.9***	**7.6 ± 1.0****	**14.6 ± 2.5*****	0.75
700 cells	3.0 ± 0.9	4.7 ± 2.0	**5.9 ± 1.5***	**7.7 ± 0.8*****	**14.7 ± 2.5*****	0.77

SA = semi-automated scoring; FA = fully-automated scoring; ∞ = semi-automated system, Kinetic Imaging 5.5; $\in$ = semi-automated system, Perceptive IV; β = the ANOVA analysis on HePG2 cells exposed to EMS was done on transformed data (square root) to fulfill the criteria of variance homogeneity; B[*a*]P, benzo[*a*]pyrene; H_2O_2, hydrogen peroxide; EMS, ethylmethane sulfonate.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.01$.

samples. For HepG2 cells exposed to B[*a*]P, there was a statistically significant effect at the highest concentration when at least 500 cells were scored. This result indicates that it may be possible to detect compounds with a weak genotoxic potential in the comet assay at low levels of DNA damage in the range of 6% tail DNA by increasing the number of cells scored. When HepG2 cells were exposed to H_2O_2, there was a statistically significant genotoxic effect at the two highest concentrations when scoring from 100 to 700 cells. However, scoring 600 or more cells resulted in a significant genotoxic effect also at the lowest concentration, inducing low levels of DNA damage in the range of 6% tail DNA.

Caco-2 cells exposed to EMS and HepG2 cells exposed to B[*a*]P were also scored (100 cells) with the semi-automated scoring system. The results show that scoring of 100 cells produced comparable values of % tail DNA with the fully-automated and the semi-automated system. Results from both scoring systems show a concentration-dependent increase in % tail DNA in HepG2 cells treated with B[*a*]P, but none of the increases was statistically significant. In Caco-2 cells exposed to EMS, the results of both systems show a concentration-dependent effect and a statistically significant increase in % tail DNA at the highest concentration compared with controls. The R^2 values for the different experiments increased by increasing the number of cells scored. Between 100 and 700 cells scored, the R^2 values increased in the range of 1.1–1.7-fold for the five experiments.

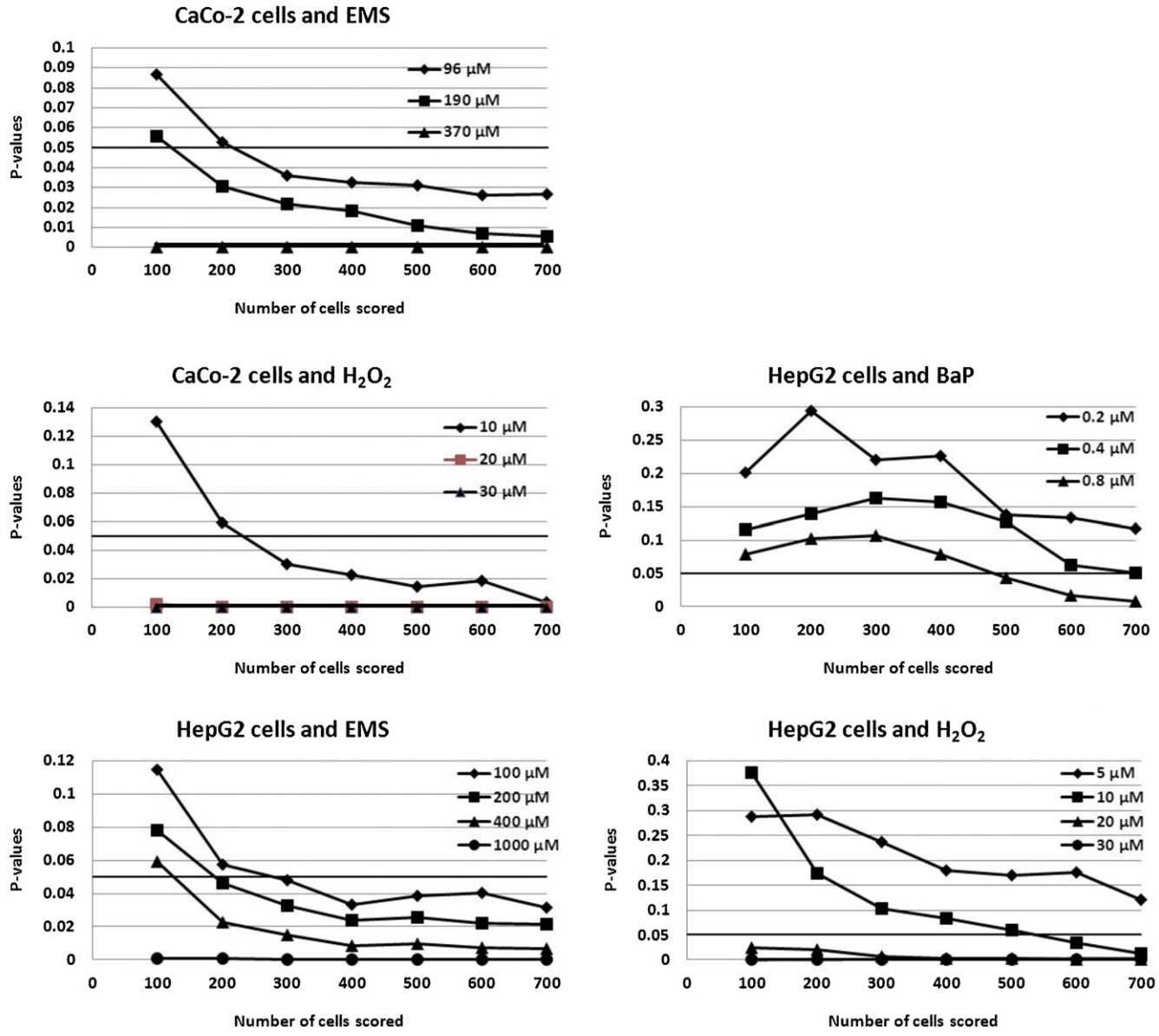

Fig. 1. Plots of *p*-values as a function of the number of cells scored by the fully-automated system for all the experiments (data from Table 1). The 0.05 *p*-value level is marked as a bold line in all plots. The *p*-values were obtained by a one-way analysis of variance, with Dunnett's test to compare the concentrations *vs* the corresponding control group.

Fig. 1 shows plots of the *p*-values for all the experiments as a function of the number of cells scored. Generally the *p*-values decreased by increasing the number of cells scored and in most situations there was a large decrease in *p*-values with increasing cell number from 100 to 400. This is also demonstrated in Table 2, which is derived from Fig. 1. The concentrations that produced a non-significant result with 100 cells scored and became significant upon scoring more cells were used in the calculations. The numbers

Table 2
The number (#) of cells required to obtain two-fold and three-fold improvements of the *p*-values calculated for 100 cells.

Cell line, compound, concentration (µM)	*p*-Value at 100 cells	# of cells to obtain a two-fold improvement in *p*-values	# of cells to obtain a three-fold improvement in *p*-values
HepG2, B[*a*]P, 0.8	0.079	500	560
HepG2, H_2O_2, 10	0.376	190	270
HepG2, EMS, 100	0.115	200	355
HepG2, EMS, 200	0.078	255	600
HepG2, EMS, 400	0.059	180	220
CaCo-2, H_2O_2, 10	0.130	195	250
CaCo-2, EMS, 96	0.087	255	540
CaCo-2, EMS, 190	0.056	235	370

Table 3
Coefficients of variances (CV%) for each concentration (n = 4–5 samples) and different number of cells scored by the fully-automatic system (CVs calculated from Table 1). A two way ANOVA analysis was performed to investigate how much the two variables (concentration and number of cells scored) could explain of the total variance in the dataset and if the effect of these two variables was statistically significant.

	Number of cells scored							Two-way ANOVA analysis
	100	200	300	400	500	600	700	
EMS (μM), Caco-2 cells								
Control	76	80	75	69	69	60	67	Number of cells: $p < 0.001$, 8% of total variation
96	82	83	77	75	64	64	65	Concentration: $p < 0.001$, 91% of total variation
190	54	50	49	45	40	39	40	
370	35	29	28	26	26	25	24	
H_2O_2 (μM), Caco-2 cells								
Control	21	44	40	10	10	8	7	Number of cells: $p = 0.013$, 29% of total variation
10	31	32	30	24	26	35	16	Concentration: $p = 0.001$, 47% of total variation
20	46	40	39	36	29	25	26	
30	23	16	12	9	10	9	9	
B[*a*]P (μM), Hep-G2 cells								
Control	67	65	58	56	53	52	44	Number of cells: $p < 0.001$, 22% of total variation
0.2	58	68	71	62	58	49	48	Concentration: $p < 0.001$, 73% of total variation
0.4	55	60	58	47	48	44	42	
0.8	32	31	37	37	28	20	18	
EMS (μM), Hep-G2 cells								
Control	109	95	90	75	70	66	62	Number of cells: $p < 0.001$, 41% of total variation
100	81	74	71	63	63	61	60	Concentration: $p < 0.05$, 38% of total variation
200	65	66	67	57	61	61	54	
400	70	72	65	64	64	62	61	
1000	85	75	73	70	69	69	69	
H_2O_2 (μM), Hep-G2 cells								
Control	48	50	43	40	37	38	32	Number of cells: $p < 0.001$, 14% of total variation
5	50	47	44	40	42	41	43	Concentration: $p < 0.001$, 80% of total variation
10	59	52	43	40	36	33	26	
20	16	19	13	8	12	13	10	
30	31	24	24	23	19	17	17	

of cells required to enhance the p-values by a factor of two and three are shown. For HepG2 cells, apart from the treatment with 0.8 μM B[*a*]P, the other concentrations required from 180 to 225 cells to obtain a two-fold improvement in p-values. For the 0.8-μM concentration of B[*a*]P, 500 cells were required. To obtain a three-fold improvement, a total cell number of 220–600 was required, with a mean value of about 400 cells.

Table 3 shows the coefficients of variance (CVs) for each concentration and for the different numbers of cells scored. An increase of cells scored resulted in a decrease of the CVs. A two-way ANOVA showed that generally the concentration explained more of the total variance in the dataset. However, the number of cells scored explained from 8 to 41% of the total variance and the effect of the number of cells scored on the CVs was also statistically significant.

4. Discussion

One way of increasing the sensitivity in the *in vitro* version of the comet assay may be to increase the number of cells scored and this issue was addressed in the present study. Using semi-automated scoring systems it is difficult to score more than 100 cells per slide for practical reasons. In contrast, the use of fully-automated scoring overcomes these practical limitations and makes it possible to score several-fold higher cell numbers. The results in Table 1 show that the sensitivity of the assay increased when scoring from 200 to 600 cells, compared with 100 cells. When increasing the number of cells scored, the absolute changes in % tail DNA were negligible, however, the standard deviations and thereby the coefficients of variance decreased. It has often been stated that one of the advantages of the comet assay is the ability to detect low levels of DNA damage and small differences in DNA damage between concentrations. Recently, it was reported that B[*a*]P was not genotoxic to HepG2 cells in the comet assay at 1 μM [14,15]. In the present study, HepG2 cells exposed to B[*a*]P showed a dose-dependent increase at low levels of DNA damage – about 6% tail DNA – and the increase was statistically significant at the highest concentration (0.8 μM) when 500 cells were scored. This indicated that it may be possible to detect compounds showing weak genotoxic potency such as B[*a*]P in the comet assay by increasing the number of cells scored. In the experiments with HepG2 cells, H_2O_2 at 10 μM induced a statistically significantly effect at low levels of DNA damage compared with control samples, when 600 cells were scored. These data suggest that an increase in sensitivity among independent samples at low levels of DNA damage can be obtained upon scoring 500–600 cells. The dataset in the present study further shows (Table 1 and Fig. 1) that at relatively low levels of DNA damage, in the range of 9–16% tail DNA, the assay sensitivity increased when scoring 200–300 cells compared with 100 cells. The increase in sensitivity can also be expressed as a decrease in p-values (Fig. 1 and Table 2) by increasing the number of cells scored, thereby increasing the statistical power.

In another study addressing the issue of number of cells scored, no significant differences were observed when 20–500 cells were scored in P388D1 leukemia cells exposed to EMS. Scoring of 5 or 10 cells was in some situations significantly different compared to scoring of 20 cells and more [6]. However, this study was based on analysis of pooled slides, whereas the present study was based on slides processed from independent experiments. A recent study of human fibroblasts exposed to methylmethane sulfonate and H_2O_2 showed that the standard errors of four parallel samples were lower when 50 or 100 cells were scored compared with 25 cells [10]. In the present study, the standard deviations decreased for independent samples and, thereby, the coefficients of variance (CVs) decreased for each concentration when the number of cells scored increased, as is illustrated in Table 3. The table shows a decrease in the CVs as a function of the number of scored cells, and the contribution from the cell number was statistically significant.

The present study demonstrated an improvement in sensitivity of the comet assay by increasing the number of cells scored. This has also been shown in the micronucleus assay when flow cytometry was used [17]. For practical reasons, it is only possible to score a large number of cells by using a fully-automated scoring system, because working with semi-automated systems is tedious and time-consuming. In the present study, capturing images by the fully-automated system took about 5 min per sample and the quantification of DNA damage was performed for all samples overnight. The processing time for scoring one sample by the semi-automated systems was about 15 min. When 100 cells were scored, the sensitivity between the fully- and semi-automated scoring systems was comparable. This is in accordance with other studies [5,6,8,10].

The present study demonstrated an increase in the assay sensitivity upon scoring more than 100 cells by a fully-automated system in *in vitro* samples. For *in vivo* samples the optimal number of cells scored could be different and recommendations for designing the comet assay with samples from rats, including experimental design issues, have been reported [11]. The comet assay has become an important tool for assessing DNA damage in exposed populations and it is the method of choice for population-based studies of environmental and occupational exposure to pollutants [2]. Increasing the sensitivity of the *in vitro* comet assay could be an important improvement in bio-monitoring studies where experimental modifications of the exposure are not possible and small differences in the level of DNA damage could be relevant. Therefore, the use of a fully-automated scoring system, allowing a higher number of cells to be scored, may be an advantage in bio-monitoring studies.

In conclusion, the important message from this study is that by increasing the number of cells scored, the coefficients of variance for each concentration decreased, resulting in a higher sensitivity of the assay. For low levels of DNA damage (below 9% tail DNA), scoring of 600 cells increased the sensitivity compared with scoring of 100 cells. For relatively low levels of DNA damage (9–16% tail DNA), scoring of 300 cells increased the sensitivity. These findings could be particularly important for bio-monitoring studies where small differences in DNA-damage level could be relevant.

Conflict of interests

There are no conflicts of interests.

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