Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 30 (3-4)
Sofia ● 2023

MORPHOLOGY 30 (3)

Original Articles

Evaluation of Different Staining Methods and Image Analyses of the Results from a Comet Assay in Human Colorectal Cancer Cells Treated with Hydrogen Peroxide

Inna Sulikovska[1], Vera Djeliova[2], Ludmil Kirazov[1], Ivaylo Ivanov[3], Mashenka Dimitrova[1]*

[1] Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2] Institute of *Molecular Biology* "*Acad. R. Tsanev*", *Bulgarian Academy* of *Sciences*, *Sofia, Bulgaria*
[3] Department of Bioorganic Chemistry and Biochemistry, Medical University, of Sofia, Bulgaria

*Corresponding author e-mail: inna_sulikovska@ukr.net

In the present study we applied comet assay to cultured *in vitro* human colorectal carcinoma cell line HT-29 in order to optimize the approach for staining and analysis of the comets after treatment with hydrogen peroxide as a DNA damaging agent. Two staining methods (fluorescent – ethidium bromide and non-fluorescent – silver staining) as well as two types of software for the analysis were evaluated. Additionally, the comets were scored visually after both types of staining. In general, we concluded that the silver staining can replace the ethidium bromide fluorescent dye, which is toxic and requires special equipment for the examination.

Key words: comet assay, ethidium bromide, silver staining, HT29 cells, DNA damage

Introduction

Oxidative DNA damage has been widely recognized as a major risk for developing deferent diseases. Hydrogen peroxide (H_2O_2) is known to produce reactive oxygen species (ROS) which are responsible for oxidative DNA lesions in animal models [1], resulting in induction and progress of neoplasms [10]. The DNA damage induced by H_2O_2 is presented by both DNA strand breaks (SBs) and alkali labile sites (ALS) which intensity is clearly dose-dependent [15]. It has been shown that under alkaline conditions, DNA loops containing breaks lose supercoiling, unwind, and are released from the nucleus to form a "comet tail" after gel electrophoresis. By applying a DNA dye, DNA SBs can be visualized and quantified by computer analysis or by visual grading [13]. Ethidium bromide (EtBr) is commonly used as a fluorescent intercalating dye that binds more efficiently to double-stranded DNA than to single stranded DNA [4]. Another possibility is the silver staining of comets. The comet assay can detect thousands lesions per cell. However, the actual percentage of tail DNA depends on the assay conditions [5]. Additionally, the methods of comet analyses can also influence the results.

The main problem in all genotoxic assays is cell death since it is also associated with degradation of DNA and so, adds to the DNA damage caused directly by the genotoxic exposure. Thus, the genotoxic agent dose should be adapted to the level of its cytotoxicity. Thresholds of cytotoxicity and cell death for comet assay reported in the literature are usually between 20% and 30%. However, the practice shows that the effect of cytotoxicity on comet assay endpoints should be assessed by a case-by-case approach rather than by adopting a predetermined threshold. Another potential source of error is the attribution of a particular tail shape to apoptosis or necrosis. For example, it has been previously accepted that the so-called "hedgehog" comets represent apoptotic cells [4]. Recently, a number of objections to this assumption are indicated [5].

There are numerous software packages to choose from, that can compute fluorescence parameters for comets selected by the operator. The most commonly used parameters are tail length, relative fluorescence intensity of head, percentage of DNA in tail and tail moment [4]. Analysis of at least 50 comets per slide has been recommended. The percentage of DNA in the comet tail represents the frequency of DNA SBs and is measured by image analysis. It is also possible to compute DNA damage from comets without sophisticated image analysis programs. The human eye is easily trained to discriminate degrees of damage according to comet appearance. The 5 classes, from 0 (no tail) to 4 (almost all DNA in tail) give sufficient resolution. If 100 comets are scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 and 400 "arbitrary units." Visual scoring is rapid as well as simple and should appeal to scientists exploring the usefulness of the technique without wanting to invest in expensive analytical equipment [4].

Image analysis from comet assays represents a significant challenge to researchers. A number of authors have compared different comet staining techniques and image analysis software to find the most optimal option for their model systems [see e.g 3, 6, 7, 9, 11].

The aim of the present study is to specify the most convenient methods for staining and analyzing the comets obtained in human colorectal carcinoma cell line HT-29 cells after the application of H_2O_2 as an inducer of oxidative DNA damage.

Materials and Methods

Hydrogen peroxide H_2O_2 (30% w/w solution in water), was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and diluted in phosphate-buffered saline (PBS, pH=7) immediately before use.

Cell culture. The HT-29 cells (human colorectal carcinoma) (ATCC, Manassas, Virginia, USA)) were cultured in Dulbecco's Modified Eagle's medium – high glucose (DMEM 4,5 g/l glucose), supplied with 10% fetal bovine serum and antibiotics in usual concentrations in a humidified atmosphere with 5% CO_2 at 37.5ºC until 90% confluence. The cells were routinely grown as monolayers in 25 cm^2 tissue culture flasks (Biologix™). Cells were detached by trypsinization (Trypsin-0.25 % EDTA).

H_2O_2 treatment. The cells were treated directly on the prepared agarose slides with encapsulated cells with 70 µl 150 µM H_2O_2 applied for 3 minutes in the dark at 4ºC by coverslips. The coverslips were removed carefully and the slides were immersed in standard lysis solution for at least 1 h in a Coplin jar at 4°C in dark.

Alkaline Comet Assay. This was performed according to a previously described procedure [5]. The slides were immersed briefly in 1% (wt/vol) LMP (low melting point) agarose, their backs were wiped and they were left on a plate overnight at room temperature (RT). The cell suspensions were counted using an automatic cell counter (Countess®, Invitrogen) and centrifuged at ~150–300g for 5 min at 4°C, washed with ice-cold PBS (phosphate buffered saline), and centrifuged again. The 45 µl of the cell suspension in PBS (~1 × 10^6cells/ml) were mixed with 105 µl 0.7% LMP agarose. Two drops (40–75µl) of the mixture were transferred to pre-coated microscope slides. The gels were covered with coverslips and kept for 5 min at 4°C in dark. The coverslips were then removed and the slides were placed in standard lysis solution (89 ml lysis stock solution: 2.5M NaCl, 100mM EDTA, 10mM Tris base, pH 10-10.5, freshly added 10 ml DMSO and 1 ml Triton X-100) for at least 1 h in a Coplin jar at 4°C in dark. The slides were transferred directly to the electrophoresis tank containing cold (4°C) solution (0.3M NaOH and 1mM Na_2EDTA, pH>13) and incubated in electrophoresis solution for 20–40 min avoiding direct light. Electrophoresis was performed at 25V (~1 V/cm) for 30 min, pH>13 at 4 °C.

The gels were neutralized by washing in cold neutralizing solution (400 mM Tris–HCl, pH 7.5) three times for 5 min. Slides were washed for 10 min in cold (4°C)

dH_2O. The gels were air-dried overnight. All steps were performed avoiding direct light.

Fluorescence staining. For staining with EtBr, 10 µg/mL aqueous solutions were added to 20–40 µL staining solution and applied to each gel. The excess EtBr was washed out by immersing the slides in 0.4 M Tris/HCl, pH 7.5 before covering them with coverslips. Stained gels can be stored overnight in the dark at RT and hydrated before scoring with 20 µL dH_2O. Comets were studied under a fluorescence microscope Leica DM 5000B (Leica microscope, Wetzlar, Germany) using appropriate filters.

Silver staining. The silver staining was performed according to the procedure [12]. The gels were fixed for 10 min in a fixing solution, containing 15% w/v trichloroacetic acid, 5% w/v zinc sulfate, and 5% glycerol. After fixation, the slides were washed three times in deionized water and dried for approximately 5 h at RT. Before silver staining, the gels were re-hydrated for 5 min in deionized water. The staining solution was prepared just before use as follows: 34 ml of the solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde) were added to 66 ml of the solution A (5% sodium carbonate). The slides were immersed for 20 – 30 min, 37°C (approximately the time required to obtain a light gray color) and placed in a shaker in small glass boxes covered with aluminum foil. After staining, the slides were washed in deionized water. The staining was stopped by immersing the slides for 5 min in 1% acetic acid solution, followed by two washes in deionized water, and were air-dried. All the glass materials used for the silver staining were pretreated with 50% nitric acid and then washed with detergents and several times with deionized water. The slides were observed and photographed using a microscope Leica DM 5000B (Leica microscope, Wetzlar, Germany).

Classification and measurement of comet parameters. For the measurement of fluorescently stained comets' parameters, the free Internet software OpenComet was used [7]. Counting of comets was done automatically. The analysis of the results was made according to the values proposed by Norozi et al. [13]. The OpenComet program allows analysis of a large number of comets, but the results were equalized to 100.

For the measurement of silver stained comets' parameters the free Internet software CaspLab was used. The comets were marked manually. For protocol validated by the parameter '% DNA in tail' in silver stained comets, we used the classification proposed by Norozi et al. [13]: 0 class (no damage) – 1-5%; 1 class (low damage) – 5–25%; 2 class (medium damage) – >25–45%; 3 class (high damage) – >45–70%; 4 class (very high damage) – >70%.

About 100 cells were evaluated per sample in visual scoring. In this system, comets are visually classified in 5 categories according to the intensity of the comet tail and head. Each comet was given a value between 0 and 4; 0 for undamaged comets and 4 for the comets with almost all DNA in their tails. DNA damage index was calculated using the following equation:

DNA damage index = 0x(n) +1x(n) +2x(n) +3x(n) +4x(n)

n = number of cells in each category

Consequently, the total score was in the range from 0 to 400 arbitrary units [2].

Statistical analysis of the results. Data were analyzed using One-way ANOVA followed by Bonferroni's post hoc test; $p < 0.05$ was accepted as the lowest level of statistical significance. The distribution of the result was checked for normality utilizing Kolmogorov-Smirnov test (GraphPad Prism software package). The data obtained were average from three independent experiments ± SD.

Results and Discussion

Presently, comet assay is widely used to evaluate the DNA damage after application of a genotoxic agent to cell cultures. It has been shown that the test can detect between ~50 and ~10,000 lesions per cell. However, the results depend substantially on the assay conditions and the method chosen for analyzing the comets. Recently, we obtained a pronounced cytotoxicity in HT-29 human tumor cell line after application of extracts from different medicinal plants endemic for our country [8, 14]. Before analyzing the possible genotoxicity of those extracts, we had to standardize the comet assay for this type of cells. For the purpose, we performed the comet assay of HT-29 cells after application of a known agent inducing oxidative DNA damage, i.e. the hydrogen peroxide. Since it was essential to apply a sub-cytotoxic concentration of H_2O_2, we used a previously recommended method for the agent application [5, 7]. We tested two types of staining – a fluorescent with EtBr and a non-fluorescent with silver nitrate. Additionally, we applied three types of analyzers – OpenComet for EtBr staining, Casplab for silver staining and also the visual analyses.

Microphotographs of EtBr and silver stained DNA of HT-29 cells for alkaline Comet assay are presented on **Fig. 1**.

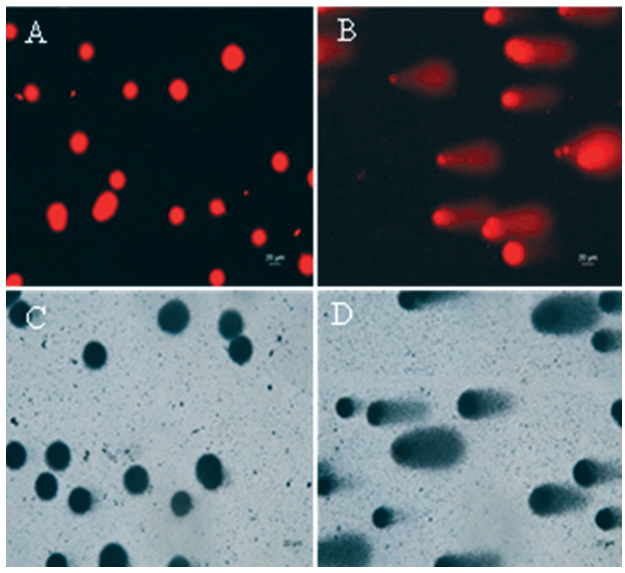

Fig. 1. Microphotographs of the stained DNA comets of HT-29 cells for alkaline Comet assay: A, B – EtBr stained non-treated cells (A) and cells treated with H_2O_2 (B); C, D – Silver stained non-treated cells (C) and cells treated with H_2O_2 (D). × 400

Typical images of the five types of comet categories obtained with the two types of staining are shown in the **Fig. 2**.

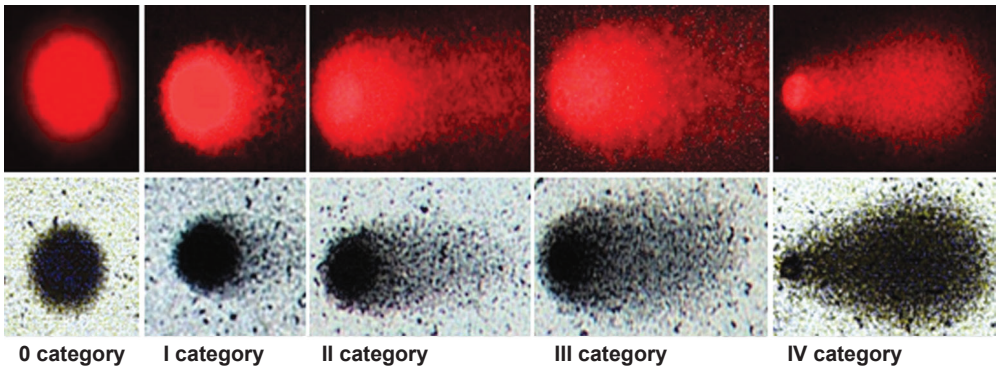

Fig. 2. Classification of the comets. Comet categories are defined by the size of the head (nucleus) and the length and intensity of the tail. 0 category – no DNA damage, I category – low DNA damage, II category – medium damage, III category – high damage, IV category – very high damage. EtBr-stained comets are represented in the top panels and silver-stained in the bottom panels.

The results obtained for DNA damage index are shown in **Fig. 3**. Data are presented as the number of cells counted in a given category/class during the visual assessment and software analysis in the range from 0 to 400 arbitrary units. In the analysis with the programs OpenComet and CaspLab, categorization of comets is

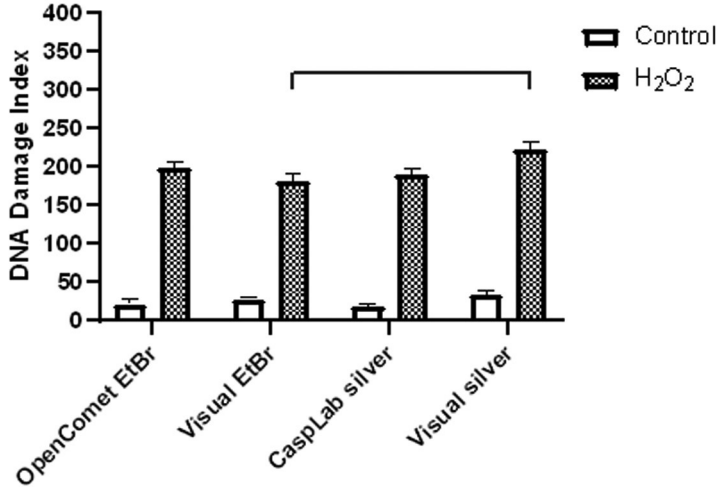

Fig. 3. Relations between the subjective visual scoring and percentage of DNA in the tail by two image analysis – CaspLab and OpenComet and the visual scoring. Each point represents the mean (± SD) of three independent counts of 100 comets.

used according to the values obtained as ‚% DNA in tail' according to the scale in [13] and calculations by the equation above [2] as explained in "Materials and methods". Statistical examination of the results are given in the figure to show that only the visual count of comets obtained after EtBr staining and the visual count of comets obtained after silver staining have a statistically significant difference (*).

The results of percentage of DNA in tail for different categories of comets are presented in **Table 1**. The obtained mean values and their deviations for each class of comets after analysis with the respective programs are presented. The table gives information about the distribution of cells in the corresponding category as a level of DNA damage.

Table 1. Percentage of DNA in tail for different categories of comets

Category	Range	OpenComet – EtBr staining		Casplab – Silver staining	
		Control	H_2O_2	Control	H_2O_2
0	<5%	2.03±1.1	3.5±1.4	0.5±0.9	0.5±0.3
I	5-25%	6.4±1.7	13.3±5.8	9.6±3.9	20.3±4.1
II	25-45%	0	35.2±6.3	0	34.9±5.1
III	45-70%	47.4±3.8	56.5±7.4	0	48.2±2.1
IV	>70%	0	85.4±8.3	0	0

The OpenComet program [7] has been developed only for the analysis of fluorescently stained comets. According to our results, it recognized perfectly the class IV (very high DNA damage) comets (**Table 1**) in this type of H_2O_2-induced DNA damage of HT-29 cells. It also classified well the comets between class III and class IV.

The CaspLab software analysis [9] performed significantly better for silver staining. The CaspLab software, compared to the other two types of analysis, has difficulty in the measuring of class IV comets (hedgehog comets – very high DNA damage) (**Table 1**) in this type of DNA damage (H_2O_2) and therefore, the results were underestimated. The program classifies comets between class II and class III very well (**Table 1**).

Visual counting is fully accepted by all researchers as one of the most accurate analyzers [4]. In the visual counting of the silver-stained preparations, the greatest degree of presence of DNA damage was obtained. This can be explained by the fact that in this type of analysis comets of type IV (very high DNA damage) are easy to classify since they have a large tail almost without a head (**Fig. 2**). However, in visual counting, the classification of comets between II and III degrees is the most subjective.

By visual counting, it is possible to make a very quick assessment of comet classes and calculate the DNA damage Index, but no more accurate data can be given regarding more detailed parameters of comet types such as: HeadArea, TailArea, HeadDNA, TailDNA, HeadDNA%, TailDNA%, HeadRadius, TailLength, CometLength, HeadMeanX, TailMeanX, TailMoment and OliveTailMoment.

A comparison between the two types of staining of preparations used in our experiments is given in **Table 2**.

Table 2. Comparison of the two staining methods: advantages and disadvantages

Staining method	Advantages	Disadvantages
EtBr Staining	Relatively fast staining, after staining with EtBr a silver staining can also be made	Toxic, requires special equipment - florescence microscope, non-permanent staining, must be analyzed within 24 hours
Silver staining	Non-toxic and requires a light microscope for analysis, permanent preparations, stained slides can be stored for years	Multi-step, slower, strong background is possible, after this staining other stainings cannot be performed

Our results for colon cancer HT-29 cells correlate well with those obtained with similar staining comparisons and assay approaches in the comet assay in previous studies with other cell types, different conditions and other agents inducing DNA damage [3, 6, 11], as well as with the same agent used by us – H_2O_2 [7, 13].

Conclusions

It can be concluded that for the analysis of DNA damage in HT-29 cells by the comet assay, both stains of the slides could be used. We can perform a faster analysis after the fluorescence staining with OpenComet, and then the same slides can be stained with silver and analyzed with the CaspLab software or by visual counting. In general, we concluded that the silver staining can replace the ethidium bromide staining, which is toxic and requires special equipment for the examination.

Acknowledgement: This work is financially supported by the National Science Fund of the Bulgarian Ministry of Education and Science, Grant Nr KP-06-N31/1.

References

1. Andreoli, C., P. Leopardi, S. Rossi, R. Crebelli. Processing of DNA damage induced by hydrogen peroxide and methyl methane sulfonate in human lymphocytes: analysis by alkaline single gel electrophoresis and cytogenetic methods. – *Mutagenesis*, **14**, 1999, 497-503.

2. Azqueta, A., S. A. S. Langie, E. Boutet-Robinet, S. Duthie, C. Ladeira, P. Møller, A. R. Collins, R. W. L. Godschalk. DNA repair as a human biomonitoring tool: Comet assay approaches. – *Mutat. Res. Rev. Mutat. Res*, **781**, 2019, 71-87.

3. Christofoletti, C. A., J. A. David, C. S. Fontanetti. Application of the comet assay in erythrocytes of Oreochromis niloticus (Pisces): A methodological comparison. – *Genet. Mol. Biol.*, **32(1)**, 2009, 155-158.
4. Collins, A. R. The comet assay for DNA damage and repair. – *Mol. Biotechnol*., **26**, 2004, 249-261.
5. Collins A., P. Møller, G. Gajski, S. Vodenková, A. Abdulwahed, et al. Measuring DNA modifications with the comet assay: a compendium of protocols. – *Nat. Protoc.*, **18(3)**, 2023, 929-989.
6. Garcia O., I. Romero, J. E. González, T. Mandina. Measurements of DNA damage on silver stained comets using free Internet software. – *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **627(2)**, 2007, 186-190.
7. Gyori, B. M., G. Venkatachalam, P. S. Thiagarajan, D. Hsu, M.-V. Clement. OpenComet: An automated tool for comet assay image analysis. – *Redox Biology*, **2**, 2014, 457-465.
8. Iliev, I., I. Ivanov, K. Todorova, D. Tasheva, M. Dimitrova. *Cotinus coggygria* non-volatile fraction affects the survival of human cultured cells. – *Acta Morphol. Anthropol*., **28(1-2)**, 2021, 19-27.
9. Konca, K., A. Lankoff, A. Banasik, H. Lisowska, T. Kuszewski, et al. A cross platform public domain PC image-analysis program for the comet assay. – *Mutat. Res.*, **534**, 2003, 15-20.
10. Kumar, N., A. Yadav, R. Gupta, N. Aggarwal. Antigenotoxic effect of *Withania somnifera* (Ashwagandha) extract against DNA damage induced by hydrogen peroxide in cultured human peripheral blood lymphocytes. – *Int. J. Curr. Microbiol. Appl. Sci.*, **5(4)**, 2016, 713-719.
11. Kyoya, T., R. Iwamoto, Y. Shimanura, M.i Terada, S. Masuda. The effect of different methods and image analyzers on the results of the in vivo comet assay. – *Genes and Environ*ment, **40**, 2018, Article Number 4.
12. Nadin, S. B., L. M. Vargas-Roig, D. R. Ciocca. A silver staining method for single-cell gel assay. – *J. Histochem Cytochem.*, **49(9),** 2001 1183-1186.
13. Noroozi, M., W. J. Angerson, M .E. J. Lean. Effects of flavonoids and Vitamin C on oxidative DNA damage to human lymphocytes. – *Am. J. Clin. Nutr.*, **67**, 1998, 1210-1218.
14. Sulikovska, I., E. Ivanova, I. Ivanov, D. Tasheva, M. Dimitrova, B. Nikolova, I. Iliev. Study on the phototoxicity and antitumor activity of plant extracts from *Tanacetum vulgare* L., *Epilobium parviflorum* Schreb., and *Geranium sanguineum* L. – *Int. J. BIOautomation*, **27(1)**, 2023, 39-50.
15. Valverde, M., J. Lozano-Salgado, P. Fortini, M.-A. Rodriguez-Sastre, E. Rojas, E. Dogliotti. Hydrogen peroxide-induced DNA damage repair through the differentiation of human adipose-derived mesenchymal stem cells. – *Stem Cells International*, **2018**, 2018, Article ID 1615497.