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Original Scientific Paper

Comparison between the Comet Assay and Fast Micromethod® for Measuring DNA Damage in HeLa Cells

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The sensitivity and precision of the single cell gel electrophoresis (Comet) assay and Fast Micromethod® for DNA damage determinations in human HeLa cell line were compared. The first assay allows analysis of DNA breaks in individual cells while the second is a rapid and convenient procedure for DNA breaks determination in cell suspensions on single microplates. Both assays detect DNA strand breaks, alkali-labile sites and transient breaks occurring at sites of ongoing repair and might be applied for the assessment of surface water genotoxic potential as well as for clinical use. DNA damage in HeLa cells was induced by different doses of γ-rays generated by Cs^{137} (8 to 500 cGy), UV-C light (10 to 1000 J m^{-2}) and by different concentrations of 4-nitroquinoline-*N*-oxide (0.026–2.6 μmol dm^{-3}). Gamma rays induced a dose-depended response with the average Comet tail moment values from 7 mm for the negative control to 291 mm for 200 cGy, from 6.1 to 192 mm for 500 J m^{-2} of UV-C light and from 7.1 to 238 mm for 1.0 μmol dm^{-3} of 4-nitroquinoline-*N*-oxide. The Fast Micromethod® strand scission factor varied from 0.010 for negative control to 0.701 for 500 cGy, from 0.019 to 1.196 for 1000 J m^{-2} and from 0.003 to 0.810 for 0.5 μmol

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dm^{-3} of 4-nitroquinoline-*N*-oxide. Sensitivity was the same for both methods and in the case of 4-nitroquinoline-*N*-oxide even better precision (lower variation coefficient) was achieved with the Fast Micromethod®. Since the time required for multiple analysis by the Fast Micromethod® is short (2 hours or less), its use in measuring DNA breakage in cells can be recommended for environmental genotoxicity monitoring.

Key words: Fast Micromethod®, Comet assay, gamma rays, UV-C light, 4-nitroquinoline-*N*-oxide, HeLa cells, DNA damage, environmental monitoring.

INTRODUCTION

Assessment of the genotoxic potential of surface water is one of the main tasks of environmental monitoring for the control of pollution. Deposition of genotoxic agents resulting from their continuous accumulation and impact on the environment requires development of sensitive and rapid assays to monitor their biological relevance. Estimation of genotoxic activity can be carried out by measuring the genetic endpoints, which exhibit primary DNA damage such as strand breaks. We recently reported on a simple and sensitive assay (Fast Micromethod®) that might find applications in environmental monitoring for genotoxic effects as in well as clinical use.[1] Two major advantages of the method are the minute amount of sample, cell suspensions or solid tissues, for one analysis and the time required for multiple analyses. The assay is based on the ability of a commercially available fluorochrome (PicoGreen®) to interact preferentially with dsDNA in the presence of ssDNA, RNA, and proteins, thus allowing direct measurements of DNA denaturation without sample handling or stepwise DNA separations. DNA denaturation, which starts after the pH has been raised from 10 to 12.4, is measured by the loss of the fluorescence signal. The time course and the extent of DNA denaturation is followed in a microplate fluorescence reader at room temperature for less than 1 h. In this way, single-strand breaks abasic sites and other alkali-labile sites and transient breaks as intermediates in the repair of DNA damage, can be detected.

The same types of DNA damage can be measured by the widely used single cell gel electrophoresis (Comet) assay.[2] A suspension of single eukaryotic cells is embedded in agarose, placed on microscope slides and submitted to lysis and unwinding of DNA, followed by electrophoresis at high pH, neutralization and staining with a fluorescent DNA binding dye.[3,4] Cells with increased DNA damage display an increased migration of the DNA from the nucleus (comet head) towards the anode (tail).[5] Routinely, 25 cells per one slide stained with ethidium bromide are examined using a fluorescent mi-

croscope. In order to detect the dose response of some known DNA damaging agents, at least 2 slides per one dose have to be examined, which makes 16 slides for 7 different doses of DNA damaging agents and the control sample. This makes the Comet assay time-consuming and tedious to perform.

In the environmental genotoxicity monitoring programs, many samples have to be compared in a short period of time. For example, 56 samples, a total of 606 tadpoles from 18 sites in southern Ontario have been investigated for genotoxicity monitoring of small bodies of water,[6] and 600 samples from 24 sites should be performed a year for monitoring the so called »hot spots« along the Adriatic coast. Therefore, a method that is simple, fast and easy to perform is urgently needed. Of course, avoidance of health hazard chemicals, such as highly genotoxic ethidium bromide, from the working environment is appreciable too. The fluorochrome PicoGreen® (P-7581) used in the Fast Micromethod® does not contain any hazardous components above 1% or any carcinogens above 0.1%, as defined in 29 CFR 1910.1200, the OSHA Hazard Communication Standard. Therefore, the Material Safety Data Sheet is not required. Moreover, factors that might affect responses in the Comet assay are numerous: cell viability, gel concentration, cell density, lysing solution (pH and ingredients of lysing solution), alkali unwinding conditions (pH and duration), electrophoresis conditions (pH, potential gradient, current and time duration), neutralisation, stain imaging conditions, scoring and cell selection criteria.[2] Factors that might affect responses in the Fast Miromethod® are the cell density and alkali unwinding conditions (pH, ionic strength), which depend on the cell type or tissue of the specific organism (*e. g.*, marine invertebrates) to be tested. Therefore, we conducted this investigation to compare the sensitivity and precision of the Fast Micromethod® and Comet assay determinations of DNA damage. DNA damage was induced by γ-irradiation, UV-C irradiation and 4-nitroquinoline-*N*-oxide treatment in the widely used, very well studied human HeLa cells, which give a predictive response to DNA damaging agents. In this way, we tested almost all types of the DNA damaging agents that can generate strand breaks, either directly or through metabolic activation, as expected in marine organisms.

EXPERIMENTAL

Chemicals

All the chemicals used in the experiments were of the highest analytical or molecular biology grade (Sigma, St. Louis, MO, USA). The low-melting agarose (electrophoresis purity quality) was obtained from BioRad (Richmond, CA, USA), PBS (phosphate buffered saline, calcium and magnesium free) and FCS were purchased from

Gibco-BRL (Paisley, Renfrewshire, Scotland, UK), HEPES from Roth (Karlsruhe, Germany). The fluorochrome PicoGreen® (P-7581) for dsDNA determination is a trademark of Molecular Probes, Inc. (Eugene, OR, USA).

Cell Line and Treatment

Cultures of the human HeLa cell line were grown in RPMI 1640 medium with 10 mmol dm^{-3} HEPES and 10% FCS in 5% of CO_2 at 37 °C. The cultures were passaged two times per week in a 1:100 ratio. Exponential growing cells were used for all experiments.

Cells (10^6/ml) were irradiated with different doses of γ-rays, generated by Cs^{137} (1800 Ci) in a Gammacell 2000 device (Mølsgaard Medical, Denmark) at 7.7 cGy s^{-1}, in dark at room temperature. After irradiation, cells were immediately placed at 4 °C, diluted to 3000/25 μl with TE buffer, pH = 7.4, and analysed.

Cells (10^6/ml) were also irradiated with different doses of UV-C light (254 nm) in a Stratalinker™ 1800 UV Crosslinker (Stratagene®, CA, USA), diluted, and kept as described above.

Cells (10^6/ml) were exposed to different concentrations of the model mutagens 4-nitroquinoline-*N*-oxide (NQO) in culture medium (0.026–2.6 μmol dm^{-3} together with 5 μl dimethyl sulfoxide (DMSO) /ml) for 1.5 h at 37 °C, diluted, and kept as described above.

Comet Assay

The alkaline single cell gel (Comet) assay was performed according to Singh *et al.*[5] Briefly, cells were suspended in low-melting-point agarose on a microscope slide. The slides were put in lysing buffer (2.5 mol dm^{-3} NaCl, 0.1 mol dm^{-3} EDTA, 0.01 mol dm^{-3} Tris, 1% Na sarcosinate, pH = 10.0) for 1 hour at 4 °C and then in electrophoresis buffer (0.3 mol dm^{-3} NaOH, 0.2 mol dm^{-3} EDTA) for 20 min to allow the DNA unwinding. During electrophoresis (25 V / 300 mA for 30 min), the broken DNA move towards the anode forming a Comet tail. Assays were conducted under alkaline conditions where single-strand breaks, abasic sites and other alkali-labile sites or intermediates in the base or nucleotide excision repair can also be detected. After electrophoresis, the slides were neutralised by Tris buffer (0.4 mol dm^{-3} Tris, pH = 7.5) for 5 min. DNA staining was performed by adding 60 μl ethidium bromide (20 μg ml^{-1}) to each slide. Slides were observed at 250 × magnification using a fluorescence microscope (Leica DMRB, Germany) equipped with an excitation filter N2.1 (BP 515–560) and a barrier filter L4 (LP 590). Comets (Figure 1) were analysed in detail using an automatic digital analysis system (Kinetic Imaging, Optilas, München, Germany; software package COMET 3.1) with quantitative image analysis. The results are expressed in the average extent tail moment ± standard deviation of 50 comets from two microscope slides per treatment and control. The extent tail moment is defined as a percentage of DNA in the tail × tail length /100.

Fast Micromethod®

The Fast Micromethod® assay was performed according to Batel *et al.*[1] The assay is based on the ability of fluorochrome (PicoGreen®) to interact preferentially with dsDNA in the presence of ssDNA, RNA, and proteins even in a wide range of

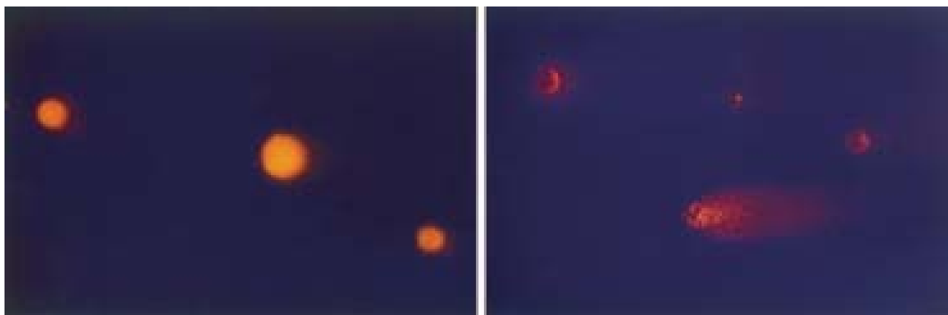

Figure 1. HeLa cells analysed by the Comet assay. Nuclei from control cells consist of a head (nucleoid core) with a minimum amount of DNA migrating into the tail region. Nuclei of the HeLa cells exposed to 0.5 μmol dm^{-3} 4-nitroquinoline-*N*-oxide (1.5 h, 37 °C) consist of a head (nucleoid core) with DNA migrating into the tail region as a result of strand breaks.

pH values and high ionic strengths. The extent of DNA denaturation at pH = 12.4 is followed directly in the microplate by measuring the fluorescence of the dsDNA-PicoGreen® complex at room temperature every 30 s for at least 20 min. In this way, the kinetics of DNA denaturation is measured. The dynamic range and sensitivity of the assay allows the quantification of as little as 25 pg ml^{-1} of dsDNA standard solutions in ordinary spectrofluorometers or in fluorescence microplate readers (25 pg in 100 μl of assay volume). The sensitivity and linear range we obtained using a Fluoroscan II reader (Labsystems) is reported.[1] Briefly, to 25 μl cell suspension (3000 cells), 25 μl lysing solution (4.5 mol dm^{-3} Urea, 0.1% SDS, 0.2 mol dm^{-3} EDTA, pH = 10.0) supplemented with PicoGreen® (20 μl of the original stock dye P-7581/ml of lysing solution) was added. The microplates were kept in the dark at room temperature for 45–60 min. DNA denaturation started after addition of 250 μl of the NaOH solution adjusted to the achieved pH = 12.4. The extent of DNA denaturation was followed directly in the microplate by measuring the fluorescence of the dsDNA-PicoGreen complex at room temperature for at least 20 min. The results were expressed as strand scission factors (SSF), calculated as the $\log_{10}$ of the ratio of the dsDNA percentage from treated and control samples, respectively, after 8 min of denaturation. For practical reasons, SSF were multiplied by –1 in graphical presentations.

RESULTS

In this paper, we compare the sensitivity of a simple method for DNA integrity estimation by measuring the extent of dsDNA denaturation at a highly alkaline pH in single microplates[1] with the sensitivity of the widely used single cell gel electrophoresis (Comet) assay.[2] The major advantage of the Fast Micromethod® is its simplicity, simultaneous measurements of multiple samples in less than 2 h, and routine automation.

DNA damage in HeLa cells was induced by different doses of γ-rays generated by Cs^{137}(8 to 500 cGy). The dose-response curves for the γ-irradiated HeLa cell line were obtained with both methods and are presented in Figure 2. Gamma rays induced a dose-dependent response with the Comet average tail moments from 7 mm for the negative control to 291 mm for 200 cGy. Fast Micromethod® average strand scission factor varied from 0.010 (97.7% dsDNA) for negative control to 0.701 (19.9% dsDNA) for 500 cGy. The effect of the minimal dose investigated of 8 cGy could be detected by both methods, suggesting their good correlation in sensitivity. In the experimental conditions applied, the sensitivity of the Comet assay is comparable with

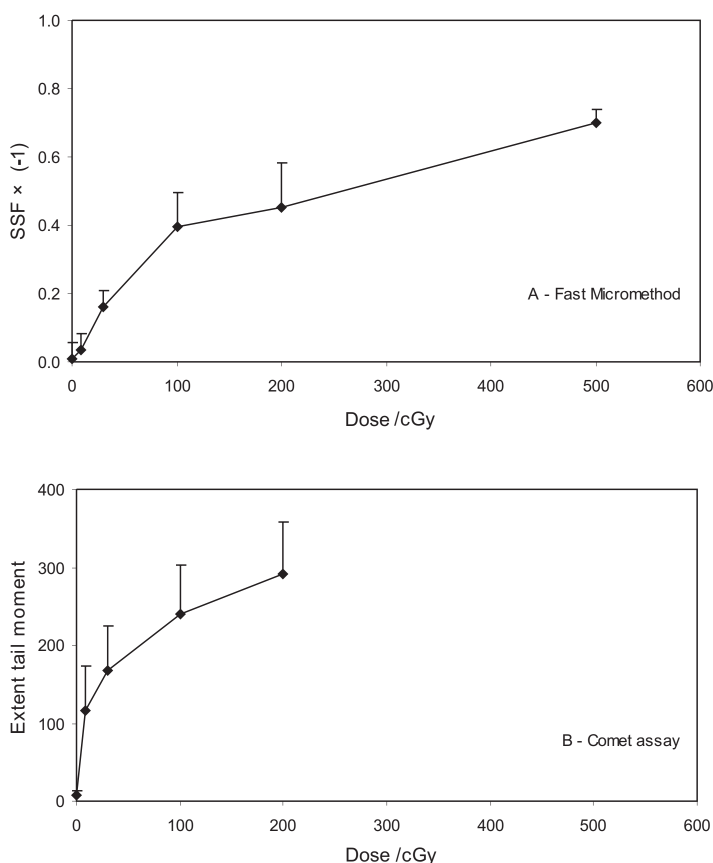

Figure 2. Strand breaks detected by a) Fast Micromethod® expressed in terms of the strand scission factor (SSF), and b) Comet assay expressed in terms of the extent tail moment in HeLa cells exposed to a range of γ-rays irradiation. Results are expressed as means ± SD (n = 6 samples for Fast Micromethod® and n = 50 comets in 2 separate cell preparations for the Comet assay).

the assay of the repair capacity of blood cells.[7] A linear dose response effect between 30 and 200 cGy was obtained and the effect of doses higher than 200 cGy could not be detected due to the low amount of DNA in comets heads. The curvilinear nature of the radiation dose-response curves for both methods could be explained by the wide range of the doses tested. Discrimination between different doses of γ-irradiation, determinated by the variation coefficient, was comparable for both methods (data not shown).

Effect of UV-C light (10 to 1000 J m^{-2}) on HeLa cell DNA is presented in Figure 3. Dose-dependent response was obtained with the Comet average tail moment values from 7.1 mm for the negative control to 192 mm for 500

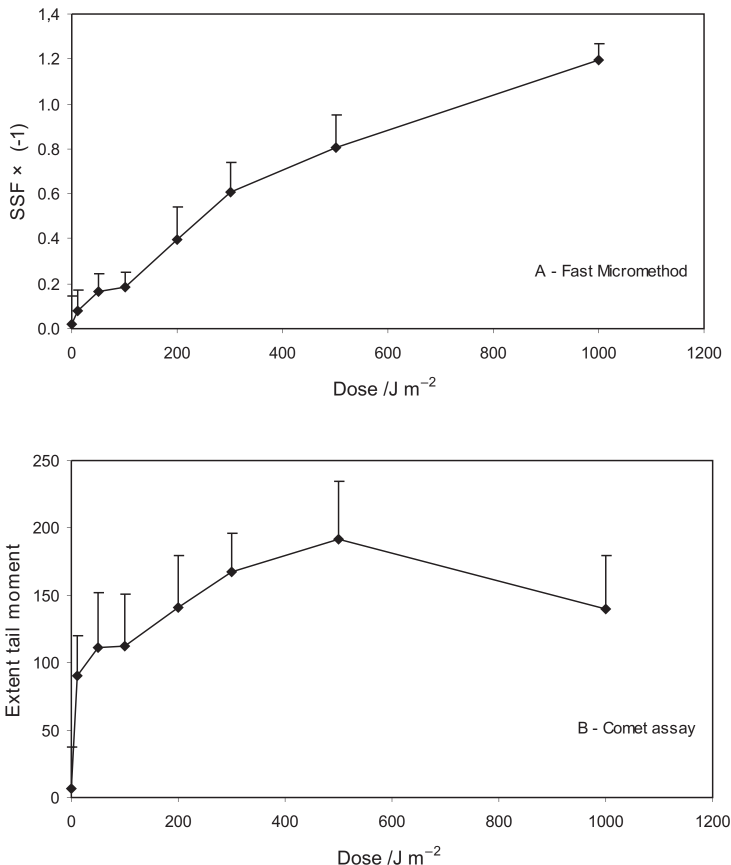

Figure 3. Strand breaks detected by a) Fast Micromethod® expressed in terms of the strand scission factor (SSF), and b) Comet assay expressed in terms of the extent tail moment in HeLa cells exposed to a range of UV-C light irradiation. Results are expressed as means ± SD (n = 6 samples for Fast Micromethod® and n = 50 comets in 2 separate cell preparations for the Comet assay).

J m^{-2} of UV-C light and with the Fast Micromethod® average strand scission factor from 0.019 (95.7% dsDNA) for negative control to 1.196 (6.4% dsDNA) for 1 000 J m^{-2}. Again, the Comet assay could not detect any additional effect of doses higher than 500 J m^{-2} of UV-C light. Both methods could not discriminate the effect of UV-C light between 50 and 100 J m^{-2}.

4-nitroquinoline-*N*-oxide induced a concentration-dependent response with the Comet average tail moment values from 5.8 mm for the negative control to 238 mm for 1.0 μmol dm^{-3} (Figure 4). Fast Micromethod® average strand scission factor varied from 0.003 (99.3% dsDNA) for negative control

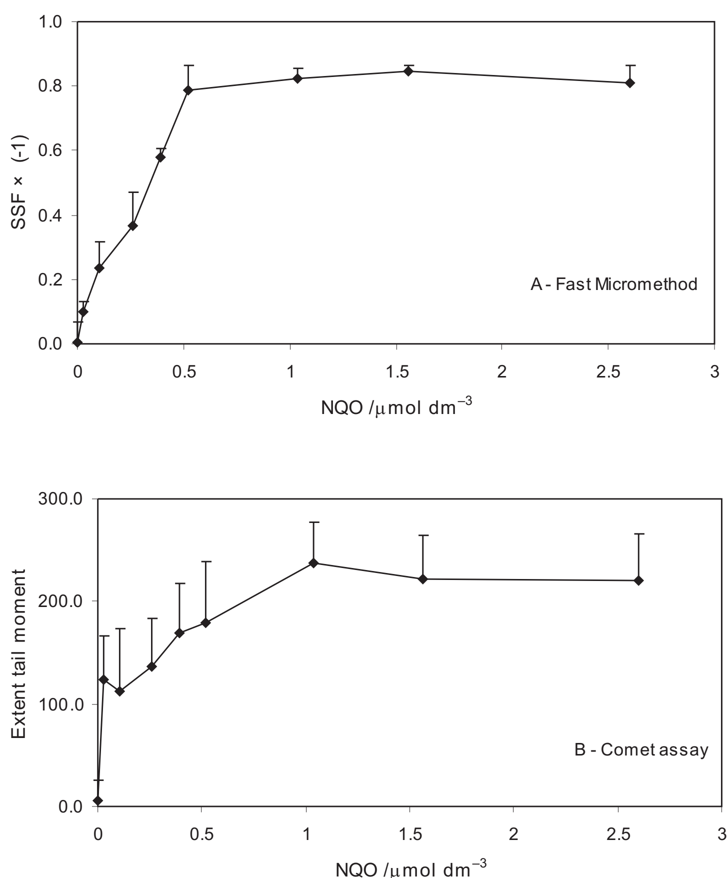

Figure 4. Strand breaks detected by a) Fast Micromethod® expressed in terms of the strand scission factor (SSF), and b) Comet assay expressed in terms of the extent tail moment in HeLa cells exposed to a range of 4-nitorquinoline-*N*-oxide. Results are expressed as means ± SD (n = 6 samples for Fast Micromethod® and n = 50 comets in 2 separate cell preparations for the Comet assay).

to 0.810 (15.5% dsDNA) for 0.5 μmol dm^{-3} NQO. The effect of the lowest concentration tested of 0.03 μmol dm^{-3} could be detected by both methods, confirming good correlation in sensitivity for both methods. Discrimination between different concentrations of NQO determined by the variation coefficient (up to 53% for »Comet« and 27% for »Fast«) was better with the Fast Micromethod®. Both methods could not detect additional effects of concentrations higher than 0.5 μmol dm^{-3} of NQO on the HeLa cells DNA. Comet assay could non detect the effect of a higher concentration due to the relatively high amount of DNA in the Comet tail. Fast Micromethod® has revealed that higher NQO concentrations cause no additional effect on HeLa DNA since SSF values remain constant.

The strand scission factor varied between 0.003–0.019 for different negative control HeLa cell lines. In this way, the background of DNA damage caused by normal cellular events was detected. However, the minimal tested doses of γ-ray, UV-C light and NQO caused an increase in SSF by 2, 4 and 4.5 times respectively, above the maximal SSF value for negative control HeLa cells. Comet results do not recognise the background DNA damage due to the absence of tail moments and remain fairly constant (5 to 7 mm).

DISCUSSION

This study aimed to establish the sensitivity and precision of two techniques used in genetic toxicology, radiation biology and medical and environmental research. These are the Comet assay[2,5,8,9] and the Fast Micromethod®.[1,10–12] Both measure single-strand breaks, abasic sites, alkali-labile sites and transient breaks as intermediates in the repair of DNA damage. Both may be used as initial indicators of general DNA damage in organism without specifying the nature of DNA modifications that lead to single-strand breaks observed under alkaline conditions. It is emphasised that it is not the strand breaks *per se* that are of health concern, but rather the DNA modifications which give rise to strand breaks and which are potentially pre-mutagenic lesions.[13] This makes both assays suitable for assessing the impact of pollution on aquatic organisms such as bullheads and carp,[14] tadpoles,[6] brown trout,[13] measured by the Comet assay, and sponge[11,15] or dab,[12] measured by the Fast Micromethod®.

To date, many different protocols have been used in the Comet assay and the variations include exposure regimes, lysis and electrophoresis techniques as well as scoring criteria.[16] The intra-laboratory and inter-laboratory comparability is therefore difficult. The sensitivity and specificity of the assay (*e.g.*, the ability to detect unknown genotoxins in the environment and the ability to discriminate between genotoxic and non-genotoxic environ-

mental samples) should be further investigated. On the other hand, the method applied for environmental genotoxicity monitoring purposes should be simple, fast and easy to perform. The comet assay, although relatively rapid, is tedious to perform (electrophoresis and microscopy of at least 20 slides per day), requires a skilled and trained person and utilises health hazard chemicals such as highly genotoxic ethidium bromide in the working environment.

These disadvantages could be overcome by the Fast Micromethod®. As shown in this paper, the results of measuring the DNA damage in HeLa cells after exposure to γ-rays, UV-C light and NQO, detected by the Fast Micromethod®, are comparable with the results obtained by the Comet assay. Sensitivity is the same for both methods, and even better precision was achieved (lower variation coefficient) in the case of NQO. Two major advantages of the method are the minute amount of sample (30 ng DNA per single weel – about 3000 cells or 25 μg of tissue) for one analysis and the time required (less than 3 hours for 96 samples in one microplate) for multiple analyses.[1] The Fast Micromethod® could be easily performed with lower taxa of sessile organisms like sponges[11,15] or mussels[17,18] since the method itself does not require removal of substances that usually interfere with DNA determinations. The samples could be collected in liquid nitrogen, transported to the laboratories, and then kept at –80 °C prior to analysis, without significant loss of DNA integrity. Considering both the results of this study and the advantages of the Fast Micromethod® mentioned above, we can recommend the Fast Micromethod® for environmental genotoxicity monitoring.

CONCLUSION

Comet assay and Fast Micromethod® showed similar sensitivity and precision concerning direct DNA damaging agents. Since the time required for multiple analysis by the Fast Micromethod® is short (2 hours or less), its use to measure genotoxic damage in cells can be recommended for environmental genotoxicity monitoring.

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SAŽETAK

Usporedba tehnike Comet i Fast Micromethod® za mjerenje oštećenja DNA u HeLa stanicama

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Uspoređena je osjetljivost i preciznost tehnike Comet i Fast Micromethod® za određivanje oštećenja DNA u humanim HeLa stanicama. Tehnika Comet omogućava analizu lomova lanaca DNA u pojedinoj stanici, dok je Fast Micromethod® vrlo brza i pogodna za njihovo određivanje u suspenzijama stanica na mikropločama. Obje metode detektiraju lomove lanaca DNA, alkalno-labilna mjesta i prolazne kratkotrajne lomove lanaca DNA nastale tijekom njezina popravka. Oštećenja DNA u HeLa stanicama inducirana su različitim dozama ionizacijskog zračenja Cs^{137} (8 do 500 cGy), UV-C zračenja (10 do 1000 J m^{-2}), te različitim dozama 4-nitrokinolin-*N*-oksida (0,026–2,6 μmol dm^{-3}). Ionizacijsko zračenje inducira dozno ovisni odziv, pri čemu vrijednost tzv. »Comet average tail moment« varira od 7 mm za negativnu kontrolu

do 291 mm za dozu zračenja od 200 cGy, odnosno od 7,1 do 192 mm za dozu UV-C zračenja od 500 J m^{-2}, te od 5,8 do 238 mm za 1,0 μmol dm^{-3} 4-nitrokinolin-*N*-oksida. Faktori jednostrukih lomova variraju od 0,010 za negativnu kontrolu do 0,701 za dozu zračenja od 500 cGy, zatim od 0,019 do 1,196 za dozu UV-C zračenja od 1000 J m^{-2} te od 0,003 do 0,810 za 0,5 μmol dm^{-3} dozu 4-nitrokinolin-*N*-oksida. Postignuta je ista osjetljivost za obje metode, dok je u slučaju s NQO postignuta i bolja preciznost tj. manji koeficijent varijacije. Kako je vrijeme neophodno za izvođenje Fast Micromethod® kratko (2 sata) njezina uporaba za određivanje lomova i integriteta DNA u stanicama može se preporučiti za primjenu u monitoringu genotoksičnih zagađivala u okolišu.