Chapter 9

Comet Assay in Cancer Chemoprevention

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Abstract

The comet assay can be useful in monitoring DNA damage in single cells caused by exposure to genotoxic agents, such as those causing air, water, and soil pollution (e.g., pesticides, dioxins, electromagnetic fields) and chemo- and radiotherapy in cancer patients, or in the assessment of genoprotective effects of chemopreventive molecules. Therefore, it has particular importance in the fields of pharmacology and toxicology, and in both environmental and human biomonitoring. It allows the detection of single strand breaks as well as double-strand breaks and can be used in both normal and cancer cells. Here we describe the alkali method for comet assay, which allows to detect both single- and double-strand DNA breaks.

Key words Comet assay, DNA damage, DNA repair, Single-cell gel electrophoresis (SCGE), Genotoxic stress, Double-strand breaks (DSB), Single-strand breaks (SSB)

1 Introduction

The first method for comet assay was described in 1990 [1], when migration of the DNA from a single cell was observed as a comet, having high-molecular-weight DNA in the head and migrating fragments (i.e., damaged DNA) in the tail. The concept of tail moment was introduced as the product between the amount of DNA in the tail and the tail length, and a software which could measure it was developed. Comet assay can be used to assess the heterogeneity of DNA damage in a cell population: it has been first used in cells treated with bleomycin [2] and then to monitor the chemoresistance of human cancers and 3D cultures [3–5]. It is now well established as a method to detect DNA damage caused by chemo- and radiotherapeutic treatments on tumours [6–8], as well as to measure the extent of DNA repair in cancer cells [9–12]. Comet assay founds its application in (1) biomonitoring of DNA damaging exposure (DNA damage due to air, soil and water pollution, and to radiation exposure, such as after the Chernobyl accident) [13–18], (2) biomonitoring of phytochemical effects (such

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as green tea, cranberry juice, carotenoids) [19–21], and (3) in cancer, although some studies in lung and prostate cancer have shown no differences in the levels of DNA damage in tumours as compared to control cells [22–30].

It is important to notice that there are some limitations to the comet assay. The samples should be viable as the presence of specific lesions cannot be detected in apoptotic or necrotic cells. Tissue disaggregation should be fast but gentle in order to minimize DNA damage due to sample handling. The number of cells that can be analysed is limited, as individual scoring of the comets does not allow to analyze more than 40–50 slides per day.

Two variations of the comet assay exist: one (neutral method) can detect only double-strand breaks (DSB), while the other (alkali method, which we describe here) can detect both single- and double-strand breaks.

2 Materials

Prepare all solutions using ultrapure water (deionized water purified to attain 18 MΩ cm at 25 °C) and analytical grade reagents. Always place 70 % volume of ultrapure water in the cylinder before adding solid reagents. When they are completely dissolved, adjust the volume. Use special care when weighing and dissolving solid NaOH. Store all reagents at room temperature, unless indicated otherwise.

1. Agarose-coated microscopy slides.
   (a) One-end-frosted microscopy slides. Store them at –20 °C in their own package.
   (b) Normal melting agarose (NMA) solution: 1 % NMA in ultrapure water. Dissolve 300 mg NMA in 30 ml ultrapure water in a glass bottle and heat in microwave at low power.
2. Low melting agarose (LMA solution): 1 % LMA in DPBS. Dissolve 100 mg LMA in 10 ml DPBS and heat in microwave at low power. Prepare 90 μl aliquots in microcentrifuge tubes (*see* **Note 1**).
3. Lysis solution: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 8 g/l NaOH, 1 % Triton X-100, 10 % DMSO. Dissolve 147.1 g NaCl anhydrous, 29.22 g EDTA, 1.21 g Tris base, 8 g NaOH in 1 l ultrapure water and store at room temperature. One hour before use, add 500 μl Triton X-100 and 5 ml DMSO to 50 ml lysis solution and store at +4 °C.
4. Electrophoresis running buffer: 300 mM NaOH, 1 mM EDTA, pH 13.0. Dissolve 12 g NaOH and 2.92 g EDTA in 1 l ultrapure water. pH will be 13.0. Store at room temperature.

5. Neutralization buffer: 0.4 M Tris-Cl, pH 8.0. Prepare 1 M Tris-Cl stock solution by dissolving 121 g Tris base in 1 l ultrapure water, adjust pH to 8.0 with HCl, and sterilize by autoclaving. Dilute 20 ml 1 M Tris-Cl, pH 8.0 to 50 ml with ultrapure water.
6. Propidium iodide staining solution: 20 μg/ml propidium iodide in DPBS. Dilute 1 mg/ml propidium iodide stock solution in DPBS. You will need 50 μl/slide.
7. Horizontal electrophoresis apparatus (tank and power supply).
8. Heat-block equipped with 1.5 ml tubes rack.
9. Fluorescence microscope equipped with an excitation filter of 515–560 nm, 100× magnification objective, and a CCD camera.
10. Computer and analysis software.

3 Methods

Carry out all the procedures at room temperature unless otherwise specified. The procedure cannot be paused until slides are dried (about 4–5 h from the beginning).

3.1 Treatment of Cells

Seed 5×10^4 cells in 35 mm diameter dishes on day 1. On day 2, pretreat the cells with melatonin (or another chemopreventive agent), then add the chemotherapeutic drugs of choice or irradiate with UV or γ-ray. On day 3 (24 h posttreatment), wash cells with DPBS, and then detach them with trypsin.

3.2 Preparing Agarose-Coated Slides

Remove microscopy slides from the –20 °C freezer. Cut the edge of a 1 ml pipette tip to enlarge its opening. Wipe each of the microscopy slides with a dry paper towel to remove condensation just prior to coat it with NMA (*see* **Note 2**). Hold the slide at a 45° angle onto the NMA containing glass bottle and pipette 500 μl NMA solution onto the slide, allowing excess solution to fall back into the bottle (Fig. 1). Lean it under the fume hood with the agarose-coated side facing upward. Allow the slides to dry for at least 30 min or until the agarose coating cannot be seen anymore. Mark the agarose-coated side of the slides (*see* **Note 3**).

3.3 Embedding Cells in LMA

Place a microcentrifuge tube containing LMA solution for each of your samples in a heat block previously set at 60 °C for 15 min to allow LMA to melt. Lower the temperature to 38 °C and use the aliquots only when they have reached this temperature (*see* **Note 4**). Place as many NMA-coated slides as your samples close to the heat block and mark them with samples names. In the meantime, collect cells, spin down briefly, and wash them with DPBS (200 μl for a 35 mm dish); spin down briefly and resuspend in 10 μl DPBS.

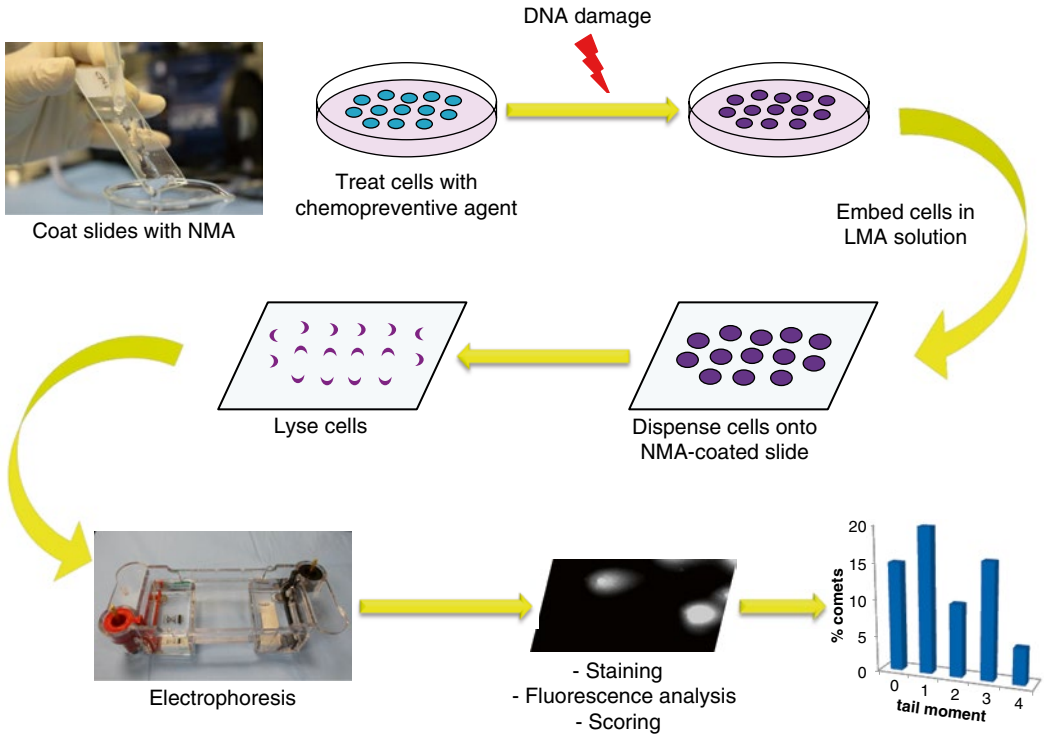

Fig. 1 Comet assay workflow. Comet assay procedure is indicated step by step

If LMA aliquots have not reached 38 °C, keep cells on ice to avoid DNA repair. Cut the edge of a 200 µl pipette tip and dispense sample into an aliquot of LMA solution. Mix and dispense immediately onto a NMA-coated slide (Fig. 1); cover with a cover slip and apply gentle pressure to spread cells. Avoid air bubbles. Repeat for all your samples. Let the cells-LMA solidify, and then remove cover slips.

3.4 Lysing Cells

Place the slides horizontally into a container and submerge them gently in cold lysis solution containing Triton X-100 and DMSO (Fig. 1). Incubate for 1 h at room temperature (*see* **Note 5**).

3.5 Equilibrating the Slides

Discard lysis solution and submerge the slides in running buffer for 20 min. Be gentle when pouring running buffer as agarose can come off the slides.

3.6 Running

Dispose the slides into a horizontal electrophoresis tank for nucleic acids and add as much running buffer as to cover the slides (Fig. 1). Run for 20 min at 300 mA and 0.6–1 V/cm length of the tank (*see* **Note 6**) to allow for proper run.

3.7 Neutralization of Samples

Gently remove the slides from the running tank and dispose them horizontally in a container. Gently wash with deionized water to remove running buffer. Submerge in neutralization buffer and incubate for 5 min. Repeat three times. Wash with deionized water.

3.8 Drying the Samples

Under a fume hood, hold the slides at a 45° angle and dispense 1 ml methanol onto the agarose with a pipet. Place horizontally and allow to completely dry (*see* **Note 7**).

3.9 Staining and Imaging

Slides can be stained by pipetting 50 μl propidium iodide solution (*see* **Note 8**) and covering with a cover slip. Samples can be visualized under a fluorescence microscope using an excitation filter of 515–560 nm. 100× magnification can be used to analyze comets (Fig. 1).

3.10 Scoring Comets

The extent of DNA damage will be estimated by both counting the comets and scoring the tail moment. Comets at the edge of the cover slips should not be taken into consideration as they usually do not run properly. In the case of melatonin, its presence strongly inhibits DNA damage hence comet tails disappear following melatonin pretreatment; thus either comets with tail or comets without tail will be observed. In the case of other pretreatments, the effect can be not so striking; thus assignment of a score to different kinds of comets is needed. There is a great number of softwares that analyze the percentage of DNA in the head or in the tail and the tail moment (i.e., the product between the amount of DNA in the tail and the tail length). Otherwise, visual scoring can be performed by measuring the tail length of each comet and assigning a score to each length, ranging from 0 for comets without tail to 4 for comets which almost lost their head and show almost all their DNA in the tail (Fig. 2).

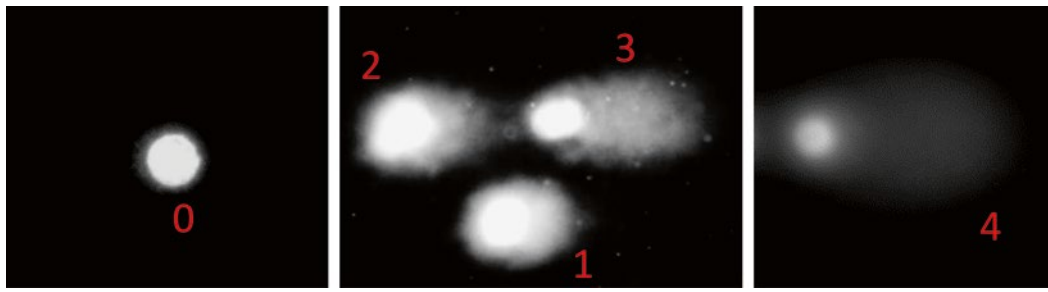

Fig. 2 Comets. Sample images are shown. Numbers in *red* indicate the score given to each comet according to the tail length

4 Notes

1. If sterile microcentrifuge tubes are used and LMA solution is dispensed when its temperature is still around 50–60 °C, aliquots can be stored for a few months. Prior to use be sure no contaminants have grown into LMA.
2. Removing condensation from microscopy slides is essential to avoid agarose comes off the slide while lysing cells or during the run.
3. Dry NMA-coated slides can be stored in a microscopy slides box at room temperature for a few months. Be sure to store them in a cool and dry place, possibly with desiccant.
4. If the cells are embedded into LMA at a temperature higher than 38 °C, DNA damage will occur.
5. Most cancer cells can be lysed by incubation in lysis solution for 1 h at room temperature. However, some cell types, such as Sk-Br-3, need a longer incubation. In these cases, incubate the slides over night at 4 °C. Such long incubation can cause NMA to come off the slides, therefore particular care should be used when handling them.
6. The length of the tank is measured from anode to cathode. In order to reach 300 mA and 0.6–1 V/cm, the volume of the running buffer should be adjusted.
7. Slides can be stored for up to 1 month before analysis. Store in a cool and dry place.
8. Slides stained with propidium iodide, as well as ethidium bromide, cannot be stored and therefore should be analyzed immediately.

References

1. Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res 122:86–94
2. Ostling O, Johanson KJ (1987) Bleomycin, in contrast to gamma irradiation, induces extreme variation of DNA strand breakage from cell to cell. Int J Radiat Biol Relat Stud Phys Chem Med 52:683–691
3. Olive PL, Banath JP (1993) Detection of DNA double-strand breaks through the cell cycle after exposure to X-rays, bleomycin, etoposide and 125IdUrd. Int J Radiat Biol 64:349–358
4. Olive PL, Banath JP (1997) Multicell spheroid response to drugs predicted with the comet assay. Cancer Res 57:5528–5533
5. Olive PL, Johnston PJ, Banath JP et al (1998) The comet assay: a new method to examine heterogeneity associated with solid tumors. Nat Med 4:103–105
6. Almeida GM, Duarte TL, Steward WP et al (2006) Detection of oxaliplatin-induced DNA crosslinks in vitro and in cancer patients using the alkaline comet assay. DNA Repair (Amst) 5:219–225
7. Dorie MJ, Kovacs MS, Gabalski EC et al (1999) DNA damage measured by the comet assay in head and neck cancer patients treated with tirapazamine. Neoplasia 1:461–467
8. Olive PL, Durand RE, Jackson SM et al (1999) The comet assay in clinical practice. Acta Oncol 38:839–844
9. Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-

induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291–298

10. Santoro R, Marani M, Blandino G et al (2012) Melatonin triggers p53Ser phosphorylation and prevents DNA damage accumulation. Oncogene 31:2931–2942
11. Santoro R, Mori F, Marani M et al (2013) Blockage of melatonin receptors impairs p53-mediated prevention of DNA damage accumulation. Carcinogenesis 34:1051–1061
12. Singh NP, Mccoy MT, Tice RR et al (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191
13. Coronas MV, Pereira TS, Rocha JA et al (2009) Genetic biomonitoring of an urban population exposed to mutagenic airborne pollutants. Environ Int 35:1023–1029
14. Moller P, Loft S (2010) Oxidative damage to DNA and lipids as biomarkers of exposure to air pollution. Environ Health Perspect 118:1126–1136
15. Mondal NK, Mukherjee B, Das D et al (2010) Micronucleus formation, DNA damage and repair in premenopausal women chronically exposed to high level of indoor air pollution from biomass fuel use in rural India. Mutat Res 697:47–54
16. Pandey AK, Bajpayee M, Parmar D et al (2005) DNA damage in lymphocytes of rural Indian women exposed to biomass fuel smoke as assessed by the Comet assay. Environ Mol Mutagen 45:435–441
17. Piperakis SM, Petrakou E, Tsilimigaki S (2000) Effects of air pollution and smoking on DNA damage of human lymphocytes. Environ Mol Mutagen 36:243–249
18. Valverde M, Rojas E (2009) Environmental and occupational biomonitoring using the Comet assay. Mutat Res 681:93–109
19. Collins AR, Olmedilla B, Southon S et al (1998) Serum carotenoids and oxidative DNA damage in human lymphocytes. Carcinogenesis 19:2159–2162
20. Duthie SJ, Jenkinson AM, Crozier A et al (2006) The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. Eur J Nutr 45:113–122
21. Glei M, Habermann N, Osswald K et al (2005) Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet assay: a biomarker model. Biomarkers 10:203–217
22. Gabelova A, Farkasova T, Gurska S et al (2008) Radiosensitivity of peripheral blood lymphocytes from healthy donors and cervical cancer patients; the correspondence of in vitro data with the clinical outcome. Neoplasma 55:182–191
23. Kosti O, Goldman L, Saha DT et al (2011) DNA damage phenotype and prostate cancer risk. Mutat Res 719:41–46
24. Lorenzo Y, Provencio M, Lombardia L et al (2009) Differential genetic and functional markers of second neoplasias in Hodgkin's disease patients. Clin Cancer Res 15:4823–4828
25. Mckenna DJ, Mckeown SR, Mckelvey-Martin VJ (2008) Potential use of the comet assay in the clinical management of cancer. Mutagenesis 23:183–190
26. Pavlov VV, Aleshchenko AV, Antoshchina MM et al (2010) Molecular-cellular characteristic of blood lymphocytes in Hodgkin lymphoma. Radiats Biol Radioecol 50:508–513
27. Santos RA, Teixeira AC, Mayorano MB et al (2010) Basal levels of DNA damage detected by micronuclei and comet assays in untreated breast cancer patients and healthy women. Clin Exp Med 10:87–92
28. Sigurdson AJ, Jones IM, Wei Q et al (2011) Prospective analysis of DNA damage and repair markers of lung cancer risk from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. Carcinogenesis 32:69–73
29. Synowiec E, Stefanska J, Morawiec Z et al (2008) Association between DNA damage, DNA repair genes variability and clinical characteristics in breast cancer patients. Mutat Res 648:65–72
30. Vasavi M, Vedicherala B, Vattam KK et al (2010) Assessment of genetic damage in inflammatory, precancerous, and cancerous pathologies of the esophagus using the comet assay. Genet Test Mol Biomarkers 14:477–482