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BRIEF REPORT

A Silver Staining Method for Single-cell Gel Assay

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SUMMARY The single-cell gel assay (comet assay) is a very useful microelectrophoretic technique for evaluation of DNA damage and repair in individual cells. Usually, the comets are visualized and evaluated with fluorescent DNA stains. This staining requires specific equipment (e.g., a high-quality fluorescence microscope), the slides must be analyzed immediately, and they cannot be stored for long periods of time. Here we describe, using human lymphocytes, some modifications of the silver staining for comets that significantly increase the sensitivity/reproducibility of the assay. This silver staining was compared with fluorescence staining and commercial silver stains. **(J Histochem Cytochem 49:1183–1186, 2001)**

KEY WORDS
comet assay
silver staining
DNA damage

THE SINGLE-CELL GEL ASSAY (also termed comet assay) is a very useful microelectrophoretic technique for evaluation of DNA damage and repair in individual cells. The technique was developed to visualize the DNA damage induced by radiation in mammalian cells (Ostling and Johanson 1984). This method has many applications in radiation biology, in estimation of oxidative damages and DNA crosslinks, in apoptosis, and in genotoxicity induced by chemical compounds (McKelvey–Martin et al. 1993). A small number of cells are immersed in an agarose gel, lysed, subjected to an electrophoretic field, and then stained with a DNA-binding fluorescent dye. The broken DNA fragments, negatively charged, migrate towards the anode and the cells can be observed under a fluorescent microscope for estimation of the damaged DNA that forms a tail like a "comet." The quantity of DNA separated from the head of the comet is proportional to the dose of irradiation.

Several versions of the comet assay are in use in research laboratories, and there are also commercial kits. There are two forms of comet assays: the neutral method for the detection of DNA double-strand breaks, and the alkaline method, which detects DNA single-strand breaks and alkali-labile lesions (Fairbairn et al. 1995). Usually, the comets are visualized and evaluated with fluorescent DNA stains, such as propidium iodide or ethidium bromide (potential carcinogens). These DNA stainings require a high-quality microscope with epifluorescent optics, a 100-W mercury lamp, a sensitive CCD camera light, and sophisticated image analysis software. Unfortunately, the slides can not be stored for long periods of time (the dye is bleached out within a day) so they must be analyzed immediately (an alternative is to store the slides with the gels and re-stain them when reexamination of the samples is needed).

We are working with lymphocytes from the peripheral blood of cancer patients to evaluate the effect of cytotoxic drugs. Cells are analyzed before and after chemotherapy. We have developed a silver staining of the comets that allows long-term storage and retrospective comparative evaluation of the cells before and after therapy. We tested a published silver staining method (Trevigen, Inc. 1999) but obtained a very low sensitivity (data not shown). Here we show the modifications introduced to the silver staining method, significantly increasing its sensitivity. We also compared our silver staining with the fluorescence method and with a silver staining commercial kit (Kizilian et al. 1999; Reinhardt–Poulin et al. 2000).

We used lymphocytes from the whole blood of a young non-smoker male donor. The lymphocytes were separated with Histopaque 1077 (Sigma; St Louis, MO) from the whole blood, washed once in PBS, centrifuged at low speed, and re-suspended in cold PBS. To prevent additional DNA damage, the cells were

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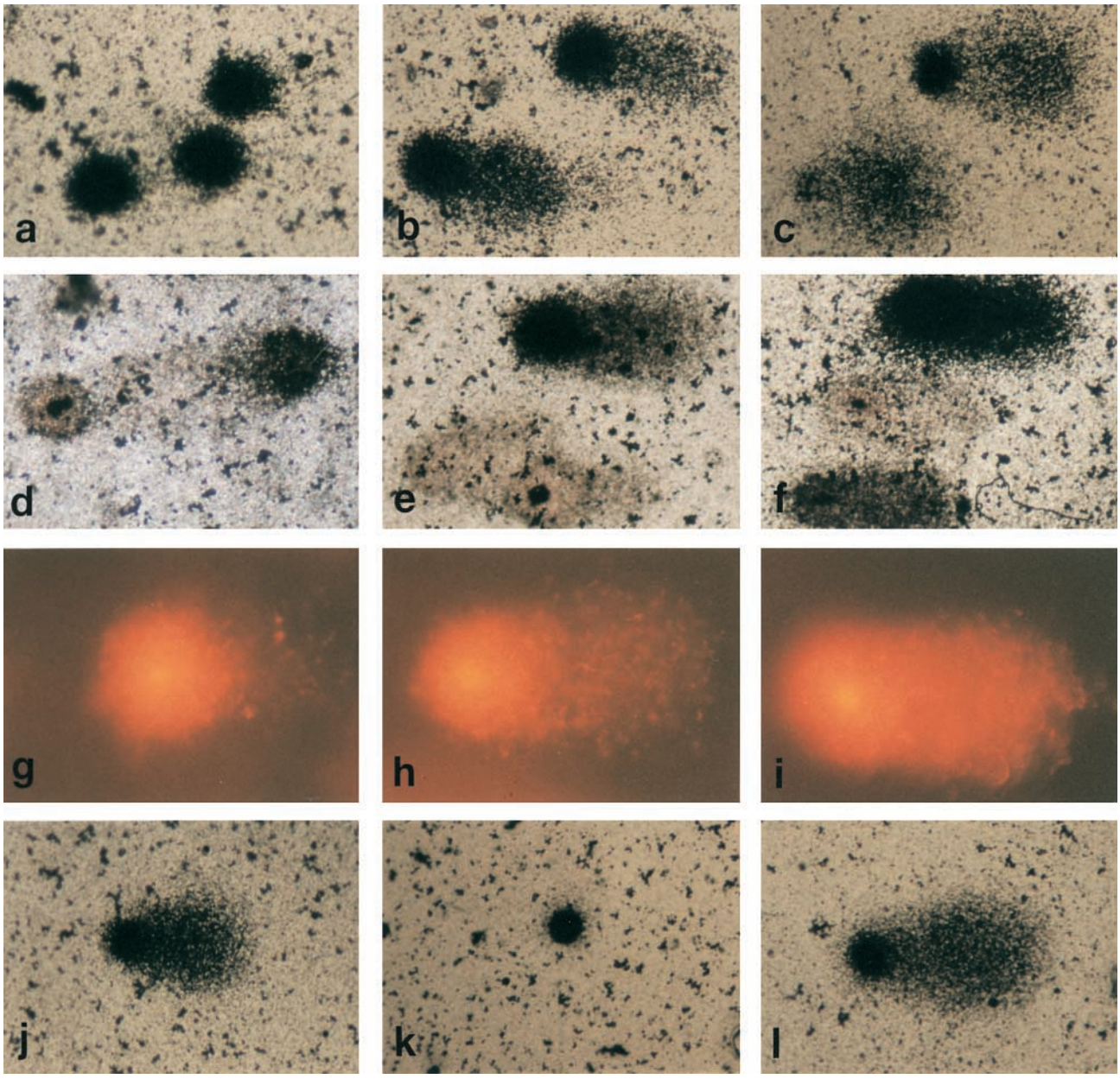

Figure 1 Staining of the comets with different procedures. (**a–c**) Silver staining as reported here (cells were treated with control buffer, 30 μM H_2O_2, or 60 μM H_2O_2, respectively). Note in **c** the apoptosis in the lower cell. (**d–f**) Silver staining using the Bio-Rad Silver Stain Plus Kit (control buffer, 30 μM H_2O_2, 60 μM H_2O_2, respectively). Note the heterogeneous staining in control cells and in the comets. (**g–i**) Fluorescence staining with propidium iodide (basal, 30 μM H_2O_2, 60 μM H_2O_2, respectively). The Trevigen silver staining is not shown because comets were not stained. (**j**) Silver staining as reported here (lymphocytes treated with 100 μM cisplatin and 60 μM H_2O_2). Note the reduction in tail length compared with **c.** (**k**) Silver staining as reported here (lymphocytes treated with 600 μM cisplatin and 60 μM H_2O_2). Here the tail is not formed and the head is reduced due to the great amount of cisplatin-induced adducts. (**l**) Silver staining as reported here. Lymphocytes were treated with 21 μM adriamycin. Original magnification ×20.

kept in the dark at 4C. Lymphocytes were treated with different DNA-damaging agents: 30 μM and 60 μM of H_2O_2, 100 μM and 600 μM of cisplatin, and 21 μM of adriamycin for 1 hr at 4C. Undamaged control cells were exposed to PBS alone. After treatment, the Alkaline Comet Assay was performed according to a described procedure (Olive et al. 1992). Cells were embedded in 1% agarose and the suspension was spread over a frosted slide. Approximately 50,000 cells were placed on each slide. After electrophoresis, the agarose gels containing the cells were placed on microscope slides and washed twice for 2 min with deionized water. The gels were dried 1 hr at room temperature (RT) and fixed for 10 min in a 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 hr at RT. Before silver staining, the gels were re-hydrated for 5 min in deionized water. The staining solution was prepared fresh before use and added to the samples very gently. The solution was prepared in the following sequence: 34 ml of 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, and 5% w/v sodium carbonate) to 66 ml of Solution A (5% sodium carbonate). The slides were immersed for 20 min (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 three or four times 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 the slides were air-dried. All the glass materials used for the silver staining were pretreated with 50% of nitric acid and then washed with detergents and several times with deionized water. The assay was performed in duplicate and we tested our method of silver staining in parallel with (a) Trevigen silver staining, (b) Bio-Rad Silver Stain Plus Kit (Hercules, CA), and (c) fluorescence (propidium iodide). For Trevigen we applied the protocol supplied in Trevigen, Inc. (1999) and for Bio-Rad silver staining we followed the manufacturer's instructions. For the propidium iodide staining, after electrophoresis the gels were rinsed in ultrapure water and stained with 2.5 μg/ml of propidium iodide dissolved in 0.1 M NaCl for 15 min. The slides were viewed and photographed using a fluorescence microscope (Nikon Opti Phot-2 UFX-IIA) (Figure 1).

Cisplatin (cis-diamminedichloroplatinum) is a chemotherapeutic agent widely used to treat several types of solid tumors. It binds to the DNA, inducing DNA interstrand crosslinks and intrastrand crosslinks implicated in cytotoxicity. Adriamycin is an anthracycline used to treat solid tumors, such as breast cancer. The cytoxicity and anti-tumor action include several mechanisms: interference with the topoisomerase II–DNA cleavable complex inducing DNA double-strand breaks, generation of free radicals, and intercalation between adjacent base pairs in the DNA. Because of the DNA crosslinks induced by cisplatin, a decrease in DNA migration during electrophoresis was observed. After incubation with cisplatin, the lymphocytes were also treated with 60 μM of H_2O_2 for 1 hr at 4C which produces fragmentation of DNA, so the decrease in migration is a measure of the damage induced by cisplatin (Figure 1).

When we compared our modified method of silver staining with the Bio-Rad Silver Stain Plus kit, we observed appreciable differences in reproducibility/sensi-

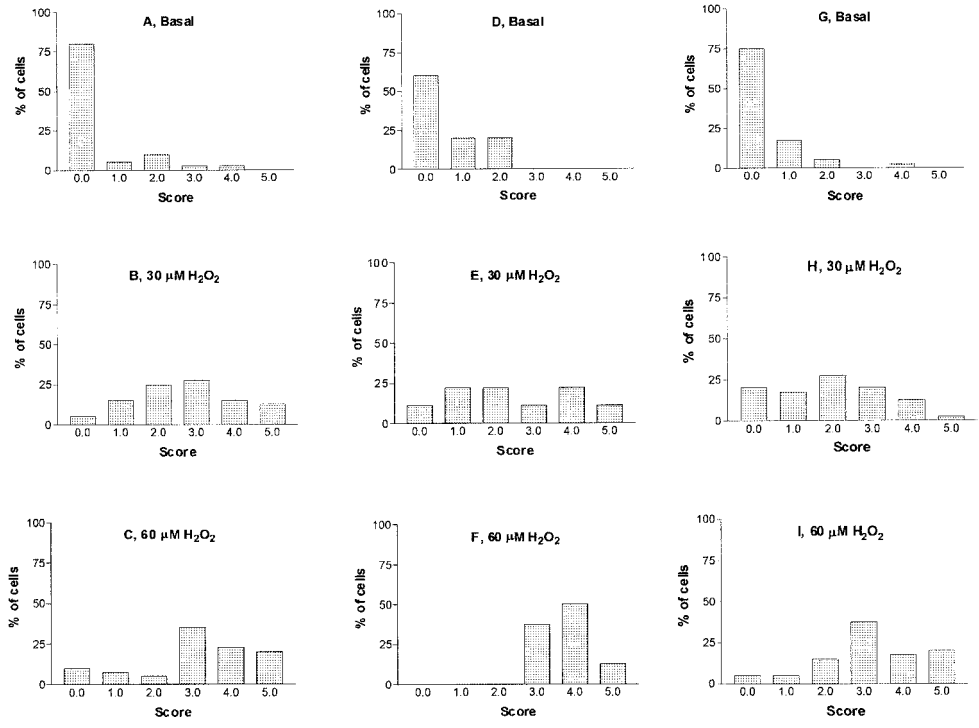

Figure 2 Schematic representation of the scores obtained after comet stainings. **(A–C)** Silver staining as reported here. **(D–F)** Fluorescence staining with propidium iodide. **(G–I)** Silver staining as reported here after fluorescent staining. There is a very good correlation between these stainings.

tivity. The agarose gels stained with the Bio-Rad kit did not show comets with a homogeneous stain, and the separation between the head and the tail of the comet was confused (Figure 1).

We also tested our silver staining after propidium iodide staining, obtaining very good results. All of the samples (at least 40 cells in each gel) were evaluated using a visual score from 0 (no damage) to 5 (total damage) (Anderson et al. 1994). Figure 2 shows the scores obtained with the different stainings. We did not find differences between silver staining and propidium iodide for all of the H_2O_2 doses. Moreover, we did not observe any difference between silver staining of the gels after propidium iodide. That is a very important observation because after the fluorescence staining the agarose gels could be dried and re-stained with silver for their documentation and archived for future analyses.

In conclusion, the comet assay provides a very simple and sensitive method to detect DNA damage. It requires a small number of cells and the results are obtained in a relatively short time. The inexpensive silver staining variation described here allows preservation of the comet assays for long periods (unlike the fluorescent dyes) and their analysis with a conventional light microscope, which is available in most laboratories. The silver staining is also very useful to keep agarose gels, previously stained with fluorescent dyes, for archival preservation of the samples.

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