CARYOLOGIA Vol. 59, no. 4: 322-325, 2006

Oxidative DNA damage induced by thinner inhalation in rats lymphocytes

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Abstract — Thinner inhalation is known to induce oxidative stress. Some studies have shown that thinner inhalation causes a decrease of antioxidants and formation of oxidation products of proteins and lipids as well as formamidopyrimidine glycoslyase (Fpg)-sensitive DNA sites. Using the comet assay and the repair-specific enzymes formamido pyrimidine glycosylase (Fpg) and endonuclease III (Endo III) to detect oxidized purines and pyrimidines, respectively, we examined the ability of thinner inhalation to induce oxidative DNA damage in rat lymphocytes. Our results show a high correlation between Fpg- and Endo III-sensitive sites. This, together with our previous results that showed a high correlation between Fpg-sensitive sites and two biomarkers of oxidative stress, suggests that these Fpg-sensitive sites correspond to oxidative damage during the first four weeks of thinner inhalation.

INTRODUCTION

Single-cell gel electrophoresis (comet assay) is a method widely used to detect DNA damage. This technique has applications in genotoxicity. The comet assay is suitable for human biomonitoring, it requires white blood cells which are obtained in a noninvasive way. This assay is simple, sensitive, reliable and economical. The most used protocol involves cells embedded in a layer of agarose on a glass slide precoated with agarose and dried, lysis with detergent and high salt, and electrophoresis in alkaline or neutral buffer.

The use of lesion-specific enzymes makes the assay more specific; digesting the nucleoids with an enzyme that recognizes a particular kind of damage. In order to detect oxidative DNA damage in rat lymphocytes obtained from rats exposed to paint thinner vapors, we have used the comet assay in conjunction with the following enzymes: endonuclease III to detect oxidized pyrimidines and formamidopyrimidine DNA glycosylase to detect 8-oxo-guanine and oxidized purines.

Paint thinner sniffing can damage the brain, kidney, liver, lung, and reproductive system (BURMISTROV *et al.* 2001; BAYDAS *et al*. 2003). One possible mechanism for tissue damage in individuals who abuse inhalants is the induction of oxidative stress, a term that denotes an imbalance between the concentration of reactive oxygen and nitrogen species. Previous studies have shown the oxidative effects of thinner inhalation on lipid peroxidation, levels of antioxidant enzymes, and glutathione depletion (HALIFEGLI *et al*. 2000; DÜNDARÖZ *et al.* 2003).

As toluene makes up 60-70% of thinner, it is possible that O2 radicals are generated from minor toluene metabolites, such as methylbenzoquinone, in the presence of NADH (MURATA *et al*. 1999). These O2 radicals can cause oxidative stress. One way to evaluate oxidative stress is to measure the concentration of the oxidation products of lipids, proteins, and DNA (DOTAN *et al.* 2004).

The objective of this work is to examine and characterize DNA damage in lymphocytes of rats exposed to thinner vapors over a 4-week period.

We have previously shown (MARTÍNEZ-ALFARO *et al*., Toxicology Letters, in press) that under our experimental conditions; thinner inhalation does not induce alkali-labile sites (ALS), single-stranded breaks (SSB), or double–stranded breaks (DSB) in DNA. Moreover, we have demonstrated the presence of Fpg-sensitive sites in DNA. However, these sites possibly correspond to either DNA oxidation or to DNA alkylation sites. Therefore, in order to correlate these Fpg-sensitive sites more directly with oxidative DNA damage, we have carried out the comet assay in conjunction with Endo III to measure oxidized pyrimidines.

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MATERIAL AND METHODS

Chemicals - Repair Endonuclease Fpg (catalog number M0240S) was obtained from New England Bio-Labs, Histopaque 1077 from the Sigma Chemical Company (product code H8889), and DAPI from Vectashield (code H-1200). All other compounds were purchased from Sigma.

Animals and experimental design - Four-week-old male Sprague-Dawley rats (weighing 100-120 g) from animal facility of the Instituto de Neurobiología UNAM were housed in polycarbonate cages. All rats were maintained in accordance with the guide for the Care and Use of Laboratory Animals (NIH). We used common thinner (purchased from PEMEX) composed of 63% toluene, 13% acetone, 10% isobutyl acetate, 7.5% isobutanol, and 6.5% butyl glycol. Thinner was vaporized into the cage with a millipore pump at constant pressure. The concentration of solvent in the exposure cage was measured with a (Drägerwerk, Germany) gas detector tube and found to be: 1000 ppm toluene. One group of 50 rats was exposed twice each day to thinner fumes for 15 minutes at room temperature in the polycarbonate cage (at this time 50% of the animals are in a narcosis state that was reversible by adequate ventilation). The rats were exposed to thinner for 2 and 4 weeks. The other group was exposed to the same conditions without solvents. At the times reported, blood samples were withdrawn from the (same) animals under anesthesia by puncture of the ophthalmic venous plexus.

Comet assay - The comet assay protocol was modified to detect oxidized bases, and it was based primarily on A. Collins' protocol (Collins *et al.* 1993). In brief, two duplicate comet slides were made for each treatment: one slide was treated with Fpg, and the other served as a control. Lymphocytes were isolated using Histopaque 1077 in accordance with the manufacturer's protocol. Then 2 x 10^4 lymphocytes with 90% viability (based on exclusion of trypan blue) were mixed with 100 µl of 0.7% agarose, and the mixture was transferred onto a frosted slide (ES 370 from Erie Scientific) pre-coated with normal melting agarose (1%). A cover slip was added, and the slide was cooled on ice to harden the agarose. For positive controls, the slides were treated with 50 µl of 2 x 10^{-5} M hydrogen peroxide for 5 minutes and then incubated 1 hour at 4°C in fresh lysis buffer (2.5 M NaCl, 0.1 M Na_2 EDTA, 0.01 M Tris-HCl, 10% DMSO, 1% Triton X-100, pH 10). For enzyme treatment, the slides were removed from lysis buffer and incubated with enzyme reaction buffer (40 mM Hepes, 0.1 M KCl, 0.5mM EDTA, and 0.2 mg/ml BSA, adjusted to pH 8 with KOH) for 10 minutes. Then either Fpg enzyme or Endo III (50 µl of 1:10^5 dilution in both cases) was added to the slides and incubated at 37°C for 35 or 30 minutes, respectively.

Slides with and without enzyme treatment were immersed in cold alkaline unwinding electrophoresis solution (0.3 M NaOH and 1 mM Na_2 EDTA in deionized water, pH 13.5) for 30 minutes and subjected to electrophoresis for 30 minutes at 25 V constant voltage. All of this procedure was carried out in the dark. The slides were stained with DAPI and analyzed by free image analysis software (AutoComet of Tritek Corporation). Triplicate samples, each containing fifty cells, were quantified for each condition. The tail moment was used as DNA damage parameter.

Statistical Analysis - All experiments were independently performed three times (150 cells in total), and differences between the mean values of the comet assay were tested for statistical significance ($p<0.05$) using the Student's t test.

RESULTS

In order to standardize the enzyme treatment for oxidative DNA damage detection, lymphocytes from three control animals were pooled and divided into six samples. One sample was used as control without treatment; the remaining samples were exposed either to H_2O_2 (20µM), to Fpg (1:10^{-5} dilution), to endo III (1:10^{-5} dilution) or were exposed simultaneously to H_2O_2 and one of the enzymes. When Fpg, Endo III, or H_2O_2 were added, there was a small but significant increase in the background level of DNA damage. However, the mean Tail Moments for the two

Table 1 — Cells without enzyme were exposed to enzyme buffer alone for 35 minutes, cells digested with Fpg were digested for 30 minutes, and those treated with Endo III were digested 35 minutes. Significant differences ($p<0.05$)*.

Treatment	Without enzyme	H_2O_2	Fpg	Fpg + H_2O_2	Endo III	Endo III + H_2O_2
Control	1.8 ± 0.3	2.4±0.2*	2.6± 0.3*	12.2±0.7*	2.8±0-3*	13.2±0-6*

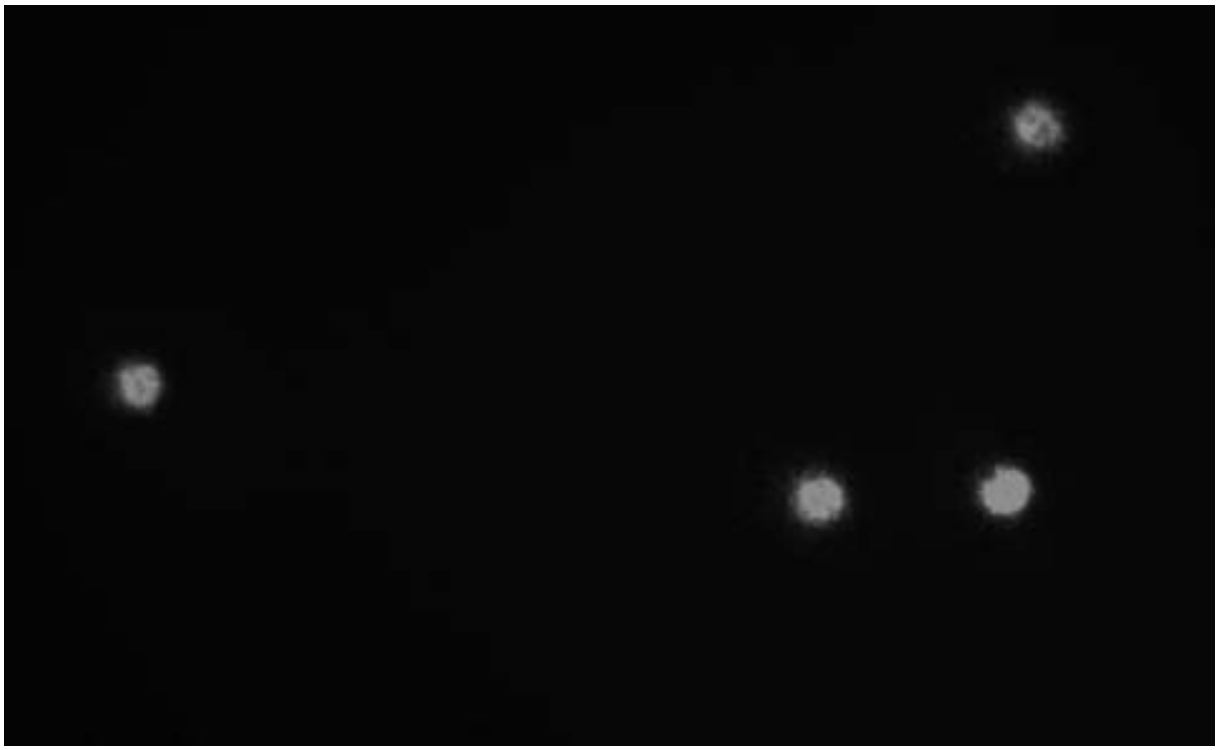

Fig. 1 — Control lymphocytes treated incubated with enzyme Endo III.

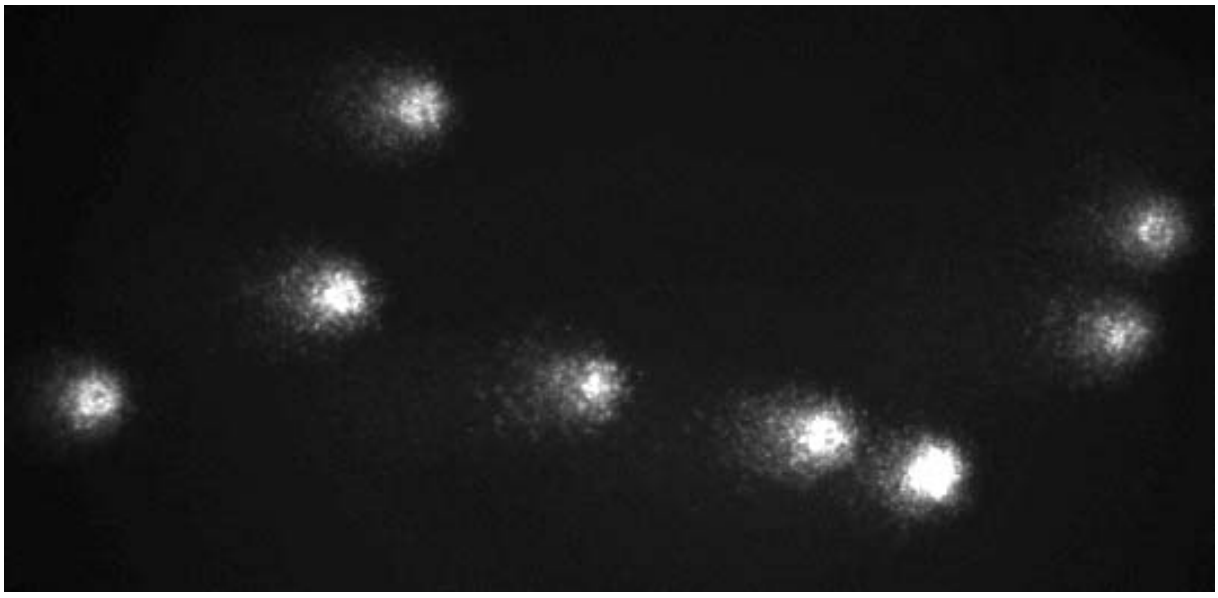

Fig. 2 — Rats lymphocytes exposed to thinner inhalation treated with Endo III enzyme.

pools of lymphocytes treated simultaneously with enzyme (Fpg or endo III) and H2O2 are four times greater than that of lymphocytes treated with either H_2O_2 or enzyme alone.

We evaluated Fpg-sensitive DNA sites and Endo III-sensitive DNA sites in lymphocytes from rats exposed to thinner fumes for two and four weeks, as shown in table 2. Samples were collected from the same six animals (three control and three treatment rats) during the treatment. Tail moment values were recorded for 50 lymphocytes per rat for each condition; the mean tail moment value for the three rats was obtained for each week examined, and the differences were

evaluated using the Student's t test. The results indicated that during all weeks of exposure, there was significant damage ($p<0.05$) in lymphocytes from rats exposed to thinner as compared to lymphocytes from control rats.

Table 2 — Lymphocytes from rats exposed to thinner inhalation for 2 or 4 weeks. Significant differences ($p<0.05$)*

Treatment	Without enzyme	Fpg	Endo III
Control	1.8± 0.3	2.6± 0.3*	2.8± 0.3*
2 weeks	2.3± 0.2	14.0± 0.8*	15.2± 0.9*
4 weeks	2.1± 0.3	9.0± 0.7*	11.2± 0.8*

DISCUSSION

We have demonstrated the presence of Endo III-sensitive DNA sites in lymphocytes exposed to thinner inhalation over a period of four weeks. In previous studies we showed a high correlation between two biomarkers of oxidative stress and Fpg-sensitives sites in DNA. However, Fpg enzyme has been reported to excise imidazole ring-opened 7-methylguanine residues in DNA (Tudek *et al*. 1998), which are formed by alkylating agents. Toluene is metabolized to a quinone methide, which can alkylate DNA (Gaikwad and Bodell 2003). The comet assay with the Fpg enzyme has been reported to detect not only oxidative damage to DNA but also the damage due to two alkylating agents (methylmethane sulfonate and ethylmethane sulfonate), although it did not detect alkylation by N-ethyl-N-nitroso urea (Speit *et al*. 2004). Therefore, we assessed Endo III-sensitive sites in lymphocytes exposed to thinner inhalation for four weeks, since these are known to be caused exclusively by oxidative damage. We used the same protocol as in our previous study (Martinez-Alfaro *et al*., Toxicology Letters in press). Our results show similar values of DNA damage detected by Fpg and Endo III enzymes, suggesting that the Fpg-sensitive sites in the lymphocytes of thinner exposed rates also correspond to oxidative DNA damage. However, we need to consider the concentration and incubation time enzyme used to detect the oxidative DNA damage induced by 20 µM H_2O_2 during 10 minutes was used in this study. Therefore, additional quantitative studies are needed to establish if purines and pyrimidines are oxidized to a similar extent by thinner inhalation.

Acknowledgements — We would like to recognize the assistance of Dr. Dorothy Pless for helpful advice. This work was supported in part by PAPIIT IN208-003-3

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