

# Preliminary Evaluation of DNA Damage Related with the Smoking Habit Measured by the Comet Assay in Whole Blood Cells

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## Abstract

The alkaline single-cell gel electrophoresis (SCGE) assay, also called the comet assay, is a rapid and simple method for the detection of DNA damage in individual cells. The objective of this study was to establish if the alkaline SCGE assay in whole blood cells gives similar results as the same method in isolated lymphocytes, because whole blood cells are simpler and more economical to use, specifically in human genotoxic biomonitoring. To validate the method, we first used mouse blood cells, because mouse is one of the most commonly used animals in genetic toxicology testing. Groups of seven CF1 male mice were given i.p. injections of relatively low doses of methyl methanesulfonate (25 mg/kg body weight), a direct acting genotoxic agent, or cyclophosphamide (50 mg/kg body weight), which requires metabolic

activation. Three, 6, 8, 12, 16, 20, and 65 hours after treatment, 5  $\mu$ L of blood were collected from each animal and were processed for the alkaline SCGE assay. On the basis of an analysis of tail moment, the results showed that this assay can detect DNA damage induced by both kinds of alkylating mutagens. We then did a preliminary study to assess the status of DNA damage in a young (19 to 23 years old) healthy population of male smokers ( $n = 6$ ) and nonsmokers ( $n = 6$ ) using the comet assay in whole blood cells. A significant difference was observed between the two groups, showing that the method is able to detect DNA damage in the smoking group despite the short time that the volunteers had actually been smoking. (Cancer Epidemiol Biomarkers Prev 2004; 13(7):1223–9)

## Introduction

The single-cell gel electrophoresis assay (SCGE), also called the comet assay, is a rapid method for the detection of DNA damage in individual cells. In the last 20 years, since its introduction by Östling and Johanson in 1984, a great number of reports have been published using the comet assay as a research tool in genotoxicity. This technique permits the detection and quantitation of DNA damage in single cells. It is simpler and faster than other conventional genotoxicity techniques, such as cytogenetics. The comet assay has also shown high sensitivity for detecting carcinogens (1), so it is a very useful tool for human biomonitoring studies (2). The SCGE assay can be done in alkaline or neutral conditions and the procedures have been reviewed elsewhere (3–5). The parameter often used to evaluate DNA damage is the “tail moment,” which is defined as the product of the tail length and the fraction of the total DNA content that is present in the tail (6).

In the present study, we first validated the use of SCGE assay in CF1 mouse whole blood cells. We correlated the posttreatment time response, measured as the tail moment, with different times of exposure to a single i.p. dose of two well-known genotoxic water-soluble compounds that have been widely used as positive controls in genotoxicity tests. Methyl methanesulfonate (MMS), a direct alkylating agent, and cyclophosphamide (CP), an agent that requires metabolic activation to induce DNA damage, were used.

We then did a preliminary study on comet induction in a small population of healthy young male smokers who smoked at least 10 cigarettes per day, using whole blood cells rather than isolated lymphocytes. It should be noted that while whole blood contains red cells, white cells, and platelets, that mature red cells do not have nuclei and, therefore, do not contribute chromosomal DNA to the results of the comet assay. White blood cells are principally represented by neutrophils and lymphocytes, with an approximate blood distribution of 55% to 60% and 25% to 35%, respectively. The other white cell types are present in very low percentages (7).

## Materials and Methods

**Chemical Reagents.** MMS (CAS no. 66-27-3), CP (CAS no. 50-15-0), low melting point agarose, and normal

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agarose were obtained from Sigma Chemical Co. (St. Louis, MO). NaCl, Na<sub>2</sub>EDTA, DMSO, Na-laurylsarcosinate, NaOH, KCl, KH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, Triton X-100, and Tris were obtained from Merck Chemical Co. (Darmstadt, Germany). Ethidium bromide was purchased from Aldrich Chemical Co. (Milwaukee, WI).

**Animals and Treatments.** Male CF1 mice, 14 weeks old, 30 to 35g body weight, were obtained from the National Health Institute of Chile. Animals were maintained with water and pellets (mouse chow Kimber, Chile) ad libitum in a controlled temperature (24°C) and humidity (40% to 50%) room with a 12-hour light-dark cycle. Before the start of the study, animals were kept under these same conditions for an acclimation period of 7 days. Groups of seven animals were given i.p. injections of 25 mg/kg body weight of MMS or 50 mg/kg body weight of CP. Both chemicals were dissolved in saline, and a maximum injection volume of 0.5 mL per animal was used. The control group (seven animals) received 0.5 mL of saline. Three, 6, 8, 12, 16, 20, and 65 hours after treatment, 5 µL of blood were collected from each animal by clipping off the last few millimeters of the tail and recovering blood with a heparinized micropipette tip.

**Humans.** All individuals answered a questionnaire that assessed their health status, dietary habits, lifestyle, and medication. These healthy selected volunteers signed a letter of consent before participation in this study. The 12 selected volunteers were University students with ages between 19 and 23 years old. Of these, 6 volunteers were nonsmokers and 6 smoked at least 10 cigarettes per day. Blood was collected with the help of a blood lancet and a heparinized micropipette tip. Three samples were collected from each individual, each one on the same day of the week during three consecutive weeks. Before each sample collection, the volunteers answered a questionnaire that assessed their habits during the last 5 days.

**Single-Cell Gel Electrophoresis.** Five microliters of mouse or human blood were mixed with 75 µL of low melting point agarose (0.5% in PBS) at 37°C. This mixture was then added to a fully frosted microscope slide coated with 110 µL of normal melting point agarose (0.6% in PBS). A 22 × 50 mm cover slip was immediately placed on top of the slide and the agarose layer was allowed to solidify for 10 minutes at 4°C. Afterwards, the cover slip was carefully removed and a second layer of low melting

point agarose without cells was added, a cover slip was applied, and the slide was held at 4°C for 5 minutes to allow the agarose layer to solidify. After removal of the cover slip, the slides were placed in lysis buffer [2.5 mol/L NaCl, 100 mmol/L Na<sub>2</sub>EDTA, 10 mmol/L Tris, 1% Na sarcosinate (pH 10)] with freshly added 1% Triton X-100 and 10% DMSO for at least 1 hour at 4°C. Subsequently, slides were placed in the electrophoresis chamber and incubated with electrophoresis alkaline buffer [300 mmol/L NaOH, 1 mmol/L Na<sub>2</sub>EDTA (pH > 13)] for 15 minutes at 4°C to allow for DNA unwinding and the expression of alkali-labile DNA damage as strand breaks. Electrophoresis was for 30 minutes at 25 V and 300 mA. The slides were then washed three times, for 5 minutes each, with neutralization buffer [0.4 mol/L Tris (pH 7.5)]. Finally, slides were stained with 50 µL of ethidium bromide (2 mg/mL), covered with a cover slip and observed at 400× magnification in a Zeiss Axioscope fluorescence microscope.

**Image and Statistical Analysis.** For each animal or human being, 20 or 50 randomly selected cells, respectively, were photographed and scanned. We excluded those cells with small heads and large fan-like tails, under the principle that they represent apoptotic cells (4, 5). The images were analyzed with the Scion Image 3b Software from the National Institute of Health, USA (<http://www.nist.gov/lispix/imlab/prelim/dnld.html>). For each cell, the length of DNA migration (tail length) was measured in micrometers from the center of the nucleus to the end of the tail. The percentage of DNA in the tail was determined by measuring the total intensity (fluorescence) in the cells, which was taken as 100%, and determining what percentage of this total intensity corresponded to the intensity measured only in the tail. The tail moment, expressed in arbitrary units, was calculated as: tail length × percentage of migrated DNA / 100.

The results of the different treatment groups were compared by use of the Student's two-tailed *t* test. A level of 0.05 was used to determine significance in all statistical analyses.

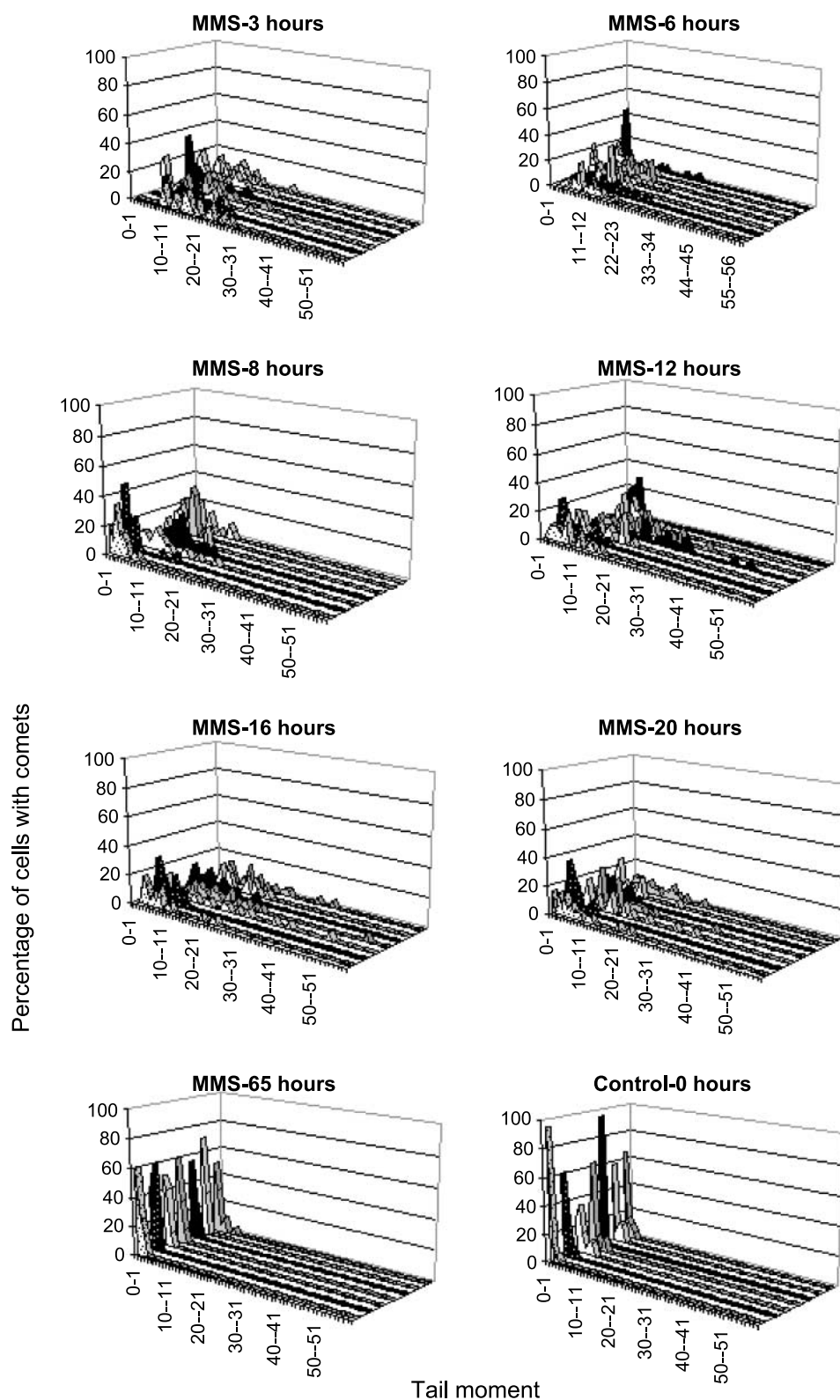
For human data, individual variability among the three samples that were collected weekly was evaluated with the Kruskal-Wallis test, and the difference between smokers and nonsmokers was evaluated by comparison of the appropriate time series data by ANOVA followed by comparison of each group by Tukey's multiple comparison test.

**Table 1. Mean tail length (±SD), mean DNA percentage in the tail (±SD), and mean tail moment (±SD) for seven CF1 mice at different posttreatment sampling times with MMS or CP**

Sampling time (h)	Tail length (µm)		DNA percentage		Tail moment	
0	0.15 ± 0.06		0.03 ± 0.02		0.54 ± 0.43	
	MMS	CP	MMS	CP	MMS	CP
3	0.38 ± 0.13*	0.13 ± 0.06	0.27 ± 0.15*	0.13 ± 0.07*	10.20 ± 4.53*	1.73 ± 1.27
6	0.39 ± 0.15*	0.22 ± 0.08	0.25 ± 0.13*	0.07 ± 0.03	9.24 ± 2.50*	1.43 ± 0.42
8	0.30 ± 0.10	0.14 ± 0.08	0.17 ± 0.09*	0.07 ± 0.03	5.20 ± 2.29*	1.12 ± 0.59
12	0.41 ± 0.12*	0.30 ± 0.09*	0.26 ± 0.12*	0.10 ± 0.04	10.97 ± 6.43*	3.42 ± 1.69*
16	0.34 ± 0.06*	0.19 ± 0.04	0.31 ± 0.06*	0.10 ± 0.04	11.39 ± 3.84*	3.37 ± 1.67*
20	0.28 ± 0.08	0.35 ± 0.10*	0.21 ± 0.06*	0.25 ± 0.11*	6.53 ± 2.54*	9.81 ± 6.70*
65	0.33 ± 0.02*	0.12 ± 0.06	0.03 ± 0.01	0.03 ± 0.01	1.09 ± 0.32	0.51 ± 0.47

NOTE: Time 0 corresponds to control group.

\*Statistically significant (*P* < 0.05).

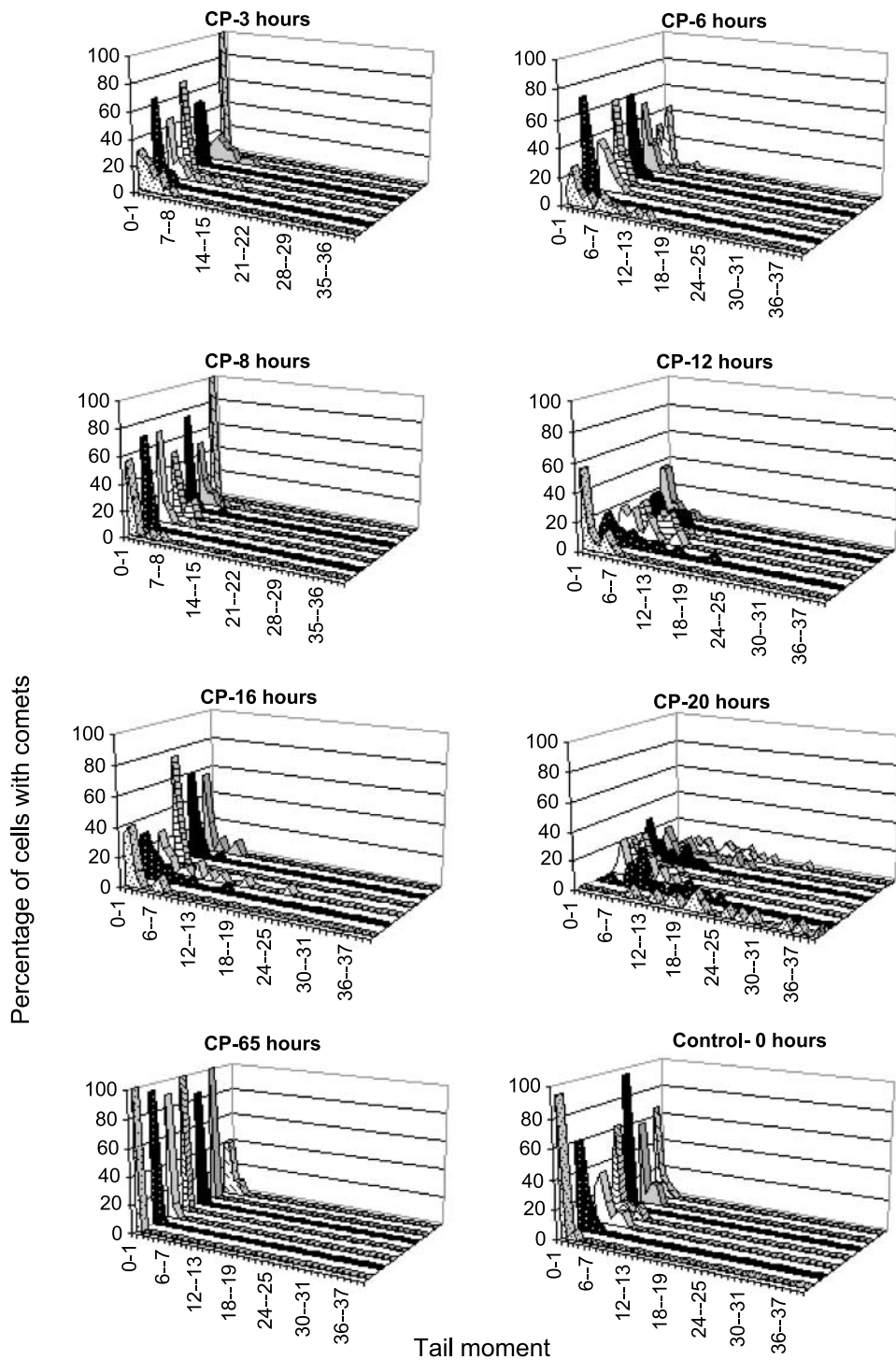


**Figure 1.** Distribution of tail moments in 20 blood cells scored in each mouse. For each sampling time, seven mice were treated with 25 mg/kg body weight of MMS.

## Results

**Comets in Mice.** The results for the induction of DNA damage by the two chemicals in mouse blood cells are

presented in Table 1. The values for tail length, DNA percentage, and tail moment are the mean of the seven mice used in each treatment time. With MMS, the mean tail moment induced at 3, 6, 8, 12, 16, and 20 hours after

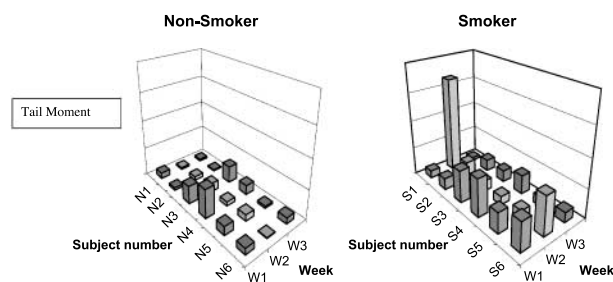


**Figure 2.** Distribution of tail moments in 20 blood cells scored in each mouse. For each sampling time, seven mice were treated with 50 mg/kg body weight of CP.

treatment is significantly higher than the mean tail moment measured in the control group. For CP-treated animals, a significantly higher tail moment was observed only at 12, 16, and 20 hours after administration of the chemical. With both chemicals, at 65 hours after treatment, the tail moment levels were not significantly different from the control group. The control group is indicated as time 0.

Figures 1 and 2 show the tail moment distribution, in the 20 cells scored for each mouse per treatment group, induced by MMS or CP, respectively. For MMS treatments at 3, 6, and 8 hours, tail moments values are distributed between 0 and 29 (Fig. 1), with 60% to 80% of cells in the 0 to 18 range. At 12 and 16 hours after treatment, tail moments ranged between 1 and 48 or 1 and 57, respectively, with 80% of cells in the 1 to 27 range





**Figure 3.** Distribution of mean tail moment scored in 50 blood cells sampled at three consecutive weeks for each volunteer, smokers and nonsmokers.

(Fig. 1). At 20 hours after treatment, tail moments are distributed between 0 and 37, with 60% of cells in the 1 and 17 range (Fig. 1).

For the CP-exposed animals analyzed 3, 6, 8, 12, and 16 hours after treatment, 95% of cells in all mice have tail moments between 0 and 15 (Fig. 2), whereas at 20 hours after treatment, tail moments are distributed between 1 and 40, with 90% of cells in the 1 to 22 tail moment range (Fig. 2).

In both the control group and the 65 hours after treatment with MMS or CP groups, tail moment distribution presented similar profiles. Tail moments ranged between 0 and 3 in control animals (Figs. 1 and 2), 0 and 7 for MMS treatment (Fig. 1), and 0 and 4 for CP treatment (Fig. 2). In both treatment groups after 65 hours, more than 90% of cells had tail moments between 0 and 3.

**Comets in Humans.** Figure 3 shows the distribution of the tail moment values for each volunteer for the three sample times, for nonsmokers and for smokers. When the data were analyzed using the Kruskal-Wallis test, differences were not significant ( $P > 0.05$ ) between the tail moment values for each individual in the three sampling times. The high value observed for smoker 1 (S1) in the second sample was correlated with his questionnaire; it showed that at this time, the volunteer had a cold episode and was consuming paracetamol (acetaminophen). When the tail moment data were analyzed from the smoking habit, a significant difference ( $P = 0.0058$ ) was observed between smokers and nonsmokers (Table 2). If we delete smoker 1 completely from the data analysis, we get values of  $0.26 \pm 0.19$  ( $n = 6$ )

versus  $0.65 \pm 0.19$  ( $n = 5$ ) for nonsmokers versus smokers; this difference is significant at  $P = 0.007$ , two-tailed  $t$  test ( $t = 3.477$ ). Thus, smoker 1 is not causing us to find a positive effect, just increasing the variance in the data. Table 2 also shows the tail moment peak value for each subject; the nonsmokers give us  $0.412 \pm 0.335$  versus a value for the smokers of  $1.425 \pm 0.989$ . We have significantly different variances between the groups, so we applied Welch's correction to our data giving us  $P = 0.0549$  (two-tailed), which is borderline significant.

## Discussion

In this study, we used the alkaline SCGE assay (comet assay) in mouse peripheral blood cells, because sampling of peripheral blood cells is easy, rapid, and inexpensive. Thus, this method can be of general applicability, especially for human biomonitoring. To establish if our method was sensitive and accurate, we used the SCGE assay in mouse blood cells treated with MMS or CP, two well-known chemical mutagens that have been widely used as positive controls in genetic toxicologic studies. Although both chemicals are alkylating agents, they present some important differences. MMS is a direct monofunctional methylating agent that forms DNA adducts by transferring alkylating groups, whereas CP, an anticancer drug, is an indirect alkylating agent which, after metabolic activation, can act as a bifunctional agent forming monoadducts and DNA-DNA cross-links (8).

The doses that we used, 25 mg/kg body weight of MMS and 50 mg/kg body weight of CP, are low doses with no reported cytotoxic effects. We had previously used, in the same mouse strain, 100 mg/kg body weight of MMS as a positive control for the micronucleus test, and no cytotoxic effect was observed (9). On the other hand, 150 mg/kg body weight of CP has been used in mice without any cytotoxic effect being reported (6).

A different response profile was observed for these two chemicals. With MMS, DNA damage, measured as the tail moment, was induced at 3 hours after treatment (Table 1 and Fig. 1). This early response is similar to that observed in other mice organs (10, 11) or in cultured cells, like L5178Y mouse lymphoma cells (12) and Chinese hamster ovary (CHO) cells (13), where DNA damage was observed immediately after MMS treatment. We observed that the response to MMS injection increased after 3 hours, reaching a maximum at 16 hours,

**Table 2. Tail moment in smokers and nonsmokers**

Nonsmokers			Smokers		
Volunteer	Tail moment	Peak value	Volunteer	Tail moment	Peak value
N1	$0.11 \pm 0.08$	$0.21 \pm 0.24$	S1	$1.24 \pm 1.83$	$3.35 \pm 2.89$
N2	$0.08 \pm 0.04$	$0.13 \pm 0.13$	S2	$0.49 \pm 0.14$	$0.65 \pm 0.50$
N3	$0.50 \pm 0.28$	$0.62 \pm 0.53$	S3	$0.64 \pm 0.43$	$1.14 \pm 0.84$
N4	$0.49 \pm 0.45$	$1.00 \pm 0.57$	S4	$0.77 \pm 0.52$	$1.33 \pm 1.20$
N5	$0.19 \pm 0.33$	$0.30 \pm 0.19$	S5	$0.46 \pm 0.23$	$0.73 \pm 0.40$
N6	$0.15 \pm 0.11$	$0.21 \pm 0.14$	S6	$0.91 \pm 0.51$	$1.35 \pm 0.88$
Mean	$0.26 \pm 0.19$	$0.41 \pm 0.34$	Mean	$0.75 \pm 0.29^*$	$1.43 \pm 0.99$

NOTE: Each tail moment value is the mean ( $\pm$ SD) of 150 blood cells in the three samples for each individual. The peak value represents the highest tail moment of each individual.

\*Statistically significant with  $P = 0.0058$ .

and then the response began to decrease. However, at 20 hours after treatment, tail moment is still significantly higher (Table 1), with a relatively high percentage of cells (60%) with high tail moments (Fig. 1). At 8 hours after treatment, we observed an apparent decrease in the mean tail moment. However, this could be an experimental artifact because two of the mice had extremely low tail moment values compared with the other five animals (Fig. 1).

For the CP treatment, we observed a weak response at the early times after treatment (3, 6, and 8 hours), and then values for tail moment began to increase (Table 1). This CP response profile is not in accordance with what others had previously reported. In isolated human blood cells, treated with S9 mix-activated CP, DNA migration was significantly increased 1 hour after the end of CP treatment (14). In an *in vivo* response study in C57BL/6 mice, which received a dose 3-fold higher than the one we used (150 mg/kg body weight versus 50 mg/kg body weight), significant DNA damage was observed in blood lymphocytes by 1 hour after the injection (15). Also, in peripheral lymphocytes of some patients undergoing chemotherapy with low doses of CP, significant DNA damage was observed by 1 hour after chemotherapy; however, the damage was most pronounced 16 to 21 hours after complete drug infusion (15).

By 65 hours after injection, the mean tail moment in the MMS- or CP-treated groups was not significantly different from the control group (Table 1), with similar tail moment distributions (Figs. 1 and 2). An explanation of these findings could be that damaged cells are removed from the blood by apoptosis or other removal processes over a period of several days. On the other hand, this recovery from DNA damage could also be due to DNA repair mechanisms. Experiments with human lymphocytes have shown that DNA damage induced by MMS persisted for a long time when cells were cultured in the presence of the repair inhibitor cytosine  $\beta$ -D-arabinofuranoside (Ara-C), whereas a complete repair of damage was observed during the first 16 hours in the absence of this inhibitor (16).

Some authors have previously used the comet assay in mouse whole blood cells with different kinds of chemicals: organophosphorus pesticides (17), potassium dichromate (18), arsenic trioxide (19), and zinc sulfate (20). In all of these experiments, the results showed a gradual decrease in tail lengths from 48 hours onwards, suggesting repair of DNA damage. Thus, for whole blood cells, it seems unnecessary to examine sampling times longer than 48 hours after treatment. This is an important consideration in the biomonitoring of human populations exposed to environmental genotoxic agents.

In summary, our results show that the alkaline SCGE assay in mouse blood cells can detect DNA damage induced by low doses of direct and indirect acting alkylating water-soluble mutagens, with an optimal sampling time at 20 hours after injection. These results are in agreement with a recent recommendation for conducting the *in vivo* alkaline comet assay (21).

Despite the fact that the comet assay has proved successful in many applications for biomonitoring of human populations, there is a controversy about its use for the evaluation of smoking habit. Some authors found a lack of adverse effect of smoking habit on DNA strand breakage using the alkaline comet assay (22), whereas

others reported statistically significant differences between tail moment response in smokers and nonsmokers (23, 24). It is important to note that the negative report was done in workers exposed to ionizing radiation in addition to the genotoxic chemicals present in the tobacco, who had high baseline levels of DNA strand breakage, whereas the positive studies were done in healthy people not exposed to other possible genotoxic compounds and, therefore, similar to our volunteers.

Although our study is a preliminary one, due the small number of subjects, it is important to note that all our study population was very young, with a brief smoking history. Despite the relatively mild insult to their DNA, the comet assay in blood cells was able to detect DNA damage in these smokers. The whole blood white cells are principally represented by neutrophils and lymphocytes, so we are using a mixture of both types of cells for detecting DNA damage. Probably, both cell types have different capacities for the bioactivation of smoke constituents and differ in their activity for DNA repair. However, the white blood cell percentage of neutrophils and lymphocytes is very stable under normal physiological conditions (7), like those present in our volunteers, so the use of this cell mixture does not substantially affect the utility of the whole blood approach. Nevertheless, it is important to understand the target cell populations for the use of the comet assay in general human biomonitoring.

We think that an important component of experimental design is a very detailed questionnaire to be answered by the subjects, because the comet assay seems to be very sensitive to different environmental factors. For example, the tail moment value of smoker 1 in the second week was increased 13-fold over the other 2-week samples (Table 2, Fig. 3). This observation was correlated with the questionnaire and was most likely to have been caused by the consumption of paracetamol (acetaminophen), because studies have reported *in vivo* genotoxicity (25, 26) and inhibitory effects of paracetamol on DNA repair in mammalian cells (27).

On the basis of our results in mice and human subjects, we can conclude that the comet assay in whole blood cells is a good assay for DNA damage detection, with the additional advantages of simplicity, economy, and speed over the comet assay in isolated lymphocytes.

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