Review

The Comet Assay as a Rapid Test in Biomonitoring Occupational Exposure to DNA-damaging Agents and Effect of Confounding Factors[1]

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Abstract

Within the last decade, the comet assay has been used with increasing popularity to investigate the level of DNA damage in terms of strand breaks and alkaline labile sites in biomonitoring studies. The assay is easily performed on WBCs and has been included in a wide range of biomonitoring studies of occupational exposures encompassing styrene, vinyl chloride, 1,3-butadiene, pesticides, hair dyes, antineoplastic agents, organic solvents, sewage and waste materials, wood dust, and ionizing radiation. Eleven of the occupational studies were positive, whereas seven were negative. Notably, the negative studies appeared to have less power than the positive studies. Also, there were poor dose-response relationships in many of the biomonitoring studies. Many factors have been reported to produce effects by the comet assay, *e.g.,* age, air pollution exposure, diet, exercise, gender, infection, residential radon exposure, smoking, and season. Until now, the use of the comet assay has been hampered by the uncertainty of the influence of confounding factors. We argue that none of the confounding factors are unequivocally positive in the majority of the studies. We recommend that age, gender, and smoking status be used as criteria for the selection of populations and that data on exercise, diet, and recent infections be registered before blood sampling. Samples from exposed and unexposed populations should be collected at the same time to avoid seasonal variation. In general, the comet assay is considered a suitable and fast test for DNA-damaging potential in biomonitoring studies.

Introduction

A wide range of methods are presently used for the detection of early biological effects of DNA-damaging agents in environmental and occupational settings. These include well-established biomarkers for chromosome damage measured by CA[3] and SCE. However, both methods are laborious and time-consuming. The micronucleus assay has found its place in biomonitoring as an assay that offers an easier technical procedure than the CA and SCE assays. Methods that detect specific adducts, such as ^{32}P postlabeling, have been developed but are used less frequently than the cytogenetic assays in human biomonitoring. It is of public interest that hazardous chemicals are removed from the environment as soon as possible. Consequently, there is a need for rapid and reliable tests that detect DNA damage of agents in different exposure circumstances. The comet assay seems to satisfy many of these criteria. It is fast, inexpensive, and requires little biological material (one drop of blood is enough for a measurement). This has made the comet assay increasingly popular during the last 10 years.

The comet assay has been developed in an empirical way, with two basically different protocols described in the literature by Singh *et al.* (1) in 1988 and Olive (2) in 1989. The former version was developed to measure low levels of strand breaks with high sensitivity, whereas the latter was optimized to detect a subpopulation of cells with varying sensitivity to drugs or radiation (3). The version of Singh *et al.* (1) has been the protocol of choice in biomonitoring studies (3). In its most simple form, the comet assay requires few steps. First, cells are embedded in agarose on a microscope slide. In the agarose, the cells and nuclear membranes are lysed, and the DNA is subjected to alkaline electrophoresis. Cellular DNA is visualized using a fluorescence microscope after staining cells with an appropriate dye. Except for these basic steps, there is currently no standard protocol for the comet assay. Different laboratories have modified the single steps to meet their particular needs. Three large reviews have been published that describe the different methodologies of the comet assay in detail (3–5). A recent publication has presented recommendations on the test procedures of the comet assay in relation to *in vitro* and *in vivo* genetic toxicology testing (6). In the present review, we have regarded these modifications as parts of the traditional comet assay procedure. In a modified protocol, an additional step of enzyme treatment is included to incise the DNA strand at the location of the damage. For instance, detection of oxidative DNA damage is possible with the FPG enzyme, which recognizes 8-oxodG and formamidopyrimidine lesions, or endonuclease III, which recognizes mostly cytosine hydrates (7). We will refer to DNA damage detected by the enzymes as FPG-sensitive sites and endonuclease III-sensitive sites.

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[3] The abbreviations used are: CA, chromosome aberration; FPG, formamidepyrimidine DNA glycosylase; MN, micronuclei; 8-oxodG, 8-oxo-2′-deoxyguanosine; SCE, sister chromatid exchange; CI, confidence interval; HPRT, hypoxanthine phosphoribosyltransferase.

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The end point measured by the traditional comet assay is a mixture of direct strand breaks and DNA damage that is converted to strand breaks by alkaline treatment. We use the term "DNA damage" to encompass all types of damage detected by the traditional comet assay. The level of DNA damage is determined largely by the length of the comet tail or by the "tail moment" (the length of the comet tail multiplied by the intensity of fluorescence in the tail). Alternatively, the level of DNA damage can be scored in arbitrary units, according to the type or grade of DNA damage. To ease the comparison between different kinds of measurements, we have calculated a mean arbitrary level of DNA damage in studies that have provided the results as type or grade of DNA damage. There is a good relationship between the level of DNA damage obtained by a visual score and that obtained by a continuous measurement (8).

The survey consists of results from 61 studies in which the comet assay has been used in biomonitoring studies. It includes a broad range of exposure circumstances, some of which address the behavior of the comet assay in healthy populations, and others that study specific confounding factors such as smoking. Many studies report the use of the comet assay to detect a DNA-damaging effect in various occupational exposure situations or after treatment with well-known agents used in chemotherapy. In most of the biomonitoring studies, the DNA damage has been detected in isolated lymphocytes or unspecified nucleated blood cells, which will not be distinguished in the present review because of occasionally missing information. We have structured the review under sections defined by the variable reported, for instance: (*a*) age; (*b*) air pollution; (*c*) diet; (*d*) gender; and (*e*) infections. One section discusses the surprising finding that exercise dramatically increases the level of DNA damage by the comet assay. Our initial efforts to use the comet assay in biomonitoring led, by coincidence, to the discovery that there was seasonal variation by the assay. We suggested that exposure to sunlight was a determinant for the seasonal variation (9), and we discuss this in one section.

Origin of DNA Damage Measured in the Comet Assay

DNA strand breaks can originate from the direct modification of DNA by chemical agents or their metabolites; from the processes of DNA excision repair, replication, and recombination; or from the process of apoptosis (10). Direct breakage of the DNA strands occurs when reactive oxygen species interact with DNA (11). When the alkaline unwinding step in the comet assay is above pH 13.1, some adducts are converted to strand breaks (*i.e.,* alkaline labile sites). The majority of publications on the comet assay state that it detects strand breaks and alkaline labile sites. An alkaline labile site can be generated by depurination of an adducted base of the nucleotide and a subsequent conversion of the abasic site to a strand break by alkaline treatment (12). However, for most DNA adducts, there is a lack of information on how chemical modifications of DNA destabilize the glycosidic bond.

In most situations, intermediate repair sites could be the most important contributors to the DNA damage detected by the comet assay. A detection of cell death by apoptosis is also possible because the hallmark of apoptosis is degradation of nuclear DNA by endonuclease enzymes that cut the DNA helix in the linker region between histone proteins (13). It has been argued that an image having a small head and a long tail (usually with >90% of the DNA in the tail) is apoptotic because the fragmentation of the DNA in apoptotic cells is so extensive that most DNA is located in the tail of the image (3). However, it is questionable whether apoptotic cells can be distinguished as a special kind of image by the comet assay.

Factors That Influence the Level of DNA Damage Detected in the Comet Assay

Intra- and interindividual variation among blood samples obtained from human donors was acknowledged in the early reports of the comet assay. In the first large review, it was recommended that information regarding the physiological and physical state of human donors should be recorded (4). There are now several studies that have described a variation in the basal level of DNA damage among healthy individuals (Table 1). The variation between the studies seems to be considerable if only the range of the lowest and the highest measurement is compared (1.2–26-fold). By calculation of the dispersion coefficient (dispersion coefficient = SD divided by the mean), the studies display a variation with the SD being 36% of the mean (95% CI, 27–46%). Holz *et al.* (14) has reported an intraindividual variation of 42% (95% CI, 18–72%), an interindividual variation of 26%, and an assay variation of 20%. It is not possible at the present time to give an estimate of the relative contributions of physiological, environmental, or assay variation to the overall variation of the comet assay. This is due in part to the fact that there have been no satisfactory descriptions of the effect of single factors or of the manner in which single factors interact with each other. Some possible factors that cause variation in the level of DNA damage in healthy untreated individuals are described below.

Age

The effect of the age of an individual in the comet assay has been assessed in statistical analyses in most of the biomonitoring studies, with apparently conflicting results. The results from the first of two cross-sectional studies of healthy Americans (age range, 25–91 years) showed no effect of age on the basal level of DNA damage (15). Likewise, large population studies from Italy comprising 200 healthy individuals (age range, 10–85 years) and 62 individuals (age range, 21–64 years) did not detect an age-related effect on the level of DNA damage (16, 17). Several studies on healthy human donors, albeit with a smaller age range, have also failed to detect an effect by age (9, 14, 18–29).

However, a few studies have detected a small effect of age by the comet assay. An American study comprising 41 individuals (age range, 24–93 years) detected a 12% increase in the basal level of DNA damage among individuals >60 years compared with individuals <60 years, which could be ascribed to a 5-fold higher content of highly damaged cells among older individuals (30). A similar effect of age was observed in hepatocytes from rats, where the subset of highly damaged cells was greater in older individuals, although the mean basal value did not increase with age (31). Also, a study of 80 individuals from Greece showed that men at the age of 55–60 years had an average of 14.5% more DNA damage than men at the age of 20–25 years (32).

Overall, the age of the individual appears to have little effect on the mean basal level of DNA damage. An interesting observation by Singh *et al.* (15) suggested that cells from older individuals have less resistance to DNA damage by *ex vivo* X-ray exposure. This suggests that the collection and processing of blood samples may produce oxidative DNA damage and that this is a more important determinant for the level of DNA damage in older individuals than in younger individuals.

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Table 1 Interindividual variation in healthy individuals by the comet assay

Population	N	Age span	Range (fold)	Mean or median (± SD)	Dispersion coefficient	Comet parameter[a]	Ref. no.
American	41	24–93	NR[b]	10.3 ± 5.91[c]	0.57	TI	30
Bulgarian	19	NR	NR	5.37 ± 2.48	0.46	TM	29
Chinese	41	NR	NR	38.89 ± 8.89	0.23	TM	59
Czech	19	20–59	1.7	6.14 ± 0.85	0.14	Tl	65
Czech	19	18–52	7	NR	NR	TM	18
Czech[d]	194	NR	4.2	5.59 ± 0.19	0.03	Tl	19
Czech	13	29–52	NR	0.6 ± 0.3	0.33	TM	78
Danish	6	40–52	7	14.25 ± 8.39	0.59	TM	9
Egyptian	10	20–57	NR	11.1 ± 4.02	0.36	TL	72
English	9	18–60	1.2	6.72 ± 0.9	0.13	TM	51
English	42	NR	NR	0.12 ± 0.3	0.25	TM	41
English	139	20–68	NR	0.61 ± 0.35	0.57	TM	20
French	11	NR	3.3	30.45 ± 12.32[e]	0.40	Arb	57
French	24	21–62	3,1	2.98 ± 0.90[f]	0.30	TM	84
Gambian	28	18–70	1.6	6.42 ± 1.69	0.26	TM	51
German[g]	10	19–37	2	69.4 ± 16.1[f]	0.23	Arb	14
German	10	25–34	4.4	0.061 ± 0.035	0.57	TN	81
German	35	25–67	NR	1.5 ± 0.5	0.30	TM	21
German	47	20–59	NR	0.041 ± 0.03	0.73	TM	22
German	9	NR	NR	2.23 ± 0.09	0.09	TM	50
German	8	23–34	2	0.046 ± 0.012	0.26	TM	85
German	8	20–39	5.7	0.19 ± 0.13	0.71	TM	86
German	8	29–34	1.2	2.1 ± 0.1	0.02	TM	48
German	13	22–70	3.1	1.5 ± 0.6	0.40	TM	87
Indian	12	NR	NR	0.49 ± 0.13	0.27	Tl	88
Italian	62	10–75	1.8	42 ± 5.35	0.13	Cl	23
Italian	12	NR	5.8	0.94 ± 0.44	0.47	TM	28
Italian	20	NR	NR	38.92 ± 8.06	0.21	Cl	24
Mexican[h]	16	≈19	14	8.76 ± 3.8	0.43	Tl	25
Mexican[i]	32	≈19	26	13.97 ± 9.32	0.67	Tl	25
Polish	40	24–65	12	24.45 ± 17.01	0.70	TM	26
Polish	41	23–52	1.4	134.9 ± 18.6	0.09	Arb	58
Slovakian	16	NR	NR	88.46 ± 32.69[f]	0.37	Arb	73
Slovakian	18	NR	NR	14.7 ± 4.4[f]	0.30	%T	64
Spanish	11	NR	NR	39.25 ± 5.9	0.15	Cl	63
Swedish	95[j]	NR	NR	39.9 ± 15.4	0.39	TM	55
Swedish	31	NR	NR	0.69 ± 0.45	0.65	TM	75
Turkish	41	NR	NR	111.39 ± 7.5	0.07	Arb	56
Turkish	8	17–43	NR	0.65 ± 0.47	1.38	Tl	27
Turkish[k]	30	19–38	1.8	248 ± 44.7	0.18	Arb	66
Turkish[k]	30	19–43	1.6	237.3 ± 30.7	0.13	Arb	61

[a] Comet parameter: Arb, arbitrary; Cl, comet length; Tl, tail length; TM, tail moment; %T, % DNA in the tail.
[b] NR, not reported.
[c] Calculated from old and young individuals combined.
[d] Calculated from the part of the study consisting of pregnant women.
[e] Calculated from samples taken at the beginning of the spraying season.
[f] The data is estimated from graphs.
[g] Based on 5–8 repeated measurements.
[h] Data reported population living in the north part of Mexico City.
[i] Data reported population living in the south part of Mexico City.
[j] Only individuals exposed to less than 200 Bq/m^3 radon.
[k] Calculated as mean arbitrary unit.

Air Pollution

Studies on the DNA-damaging effect of air pollution by the comet assay have been reported from the Czech Republic and Mexico City, two areas with some of the most severe air pollution problems in the world. One of the studies from the Czech republic investigated the effect of air pollution in postal workers and gardeners from a highly industrialized district in Bohemia (Teplice). There was an association between ambient polyaromatic hydrocarbon concentration and the DNA damage detected by the comet assay (18). The effect appeared to depend on the *GSTM1* phase 2 metabolizing enzyme genotype (*GSTM1* null genotype was the genotype associated with the highest level of DNA damage). The effect of air pollution was later questioned in a larger study of 542 people, showing that people from a region with a low level of pollution (Prachatice) and people from Teplice had a similar level of DNA damage, even when the statistical analysis was adjusted for *GSTM1* polymorphism, smoking, and ethnic background (19).

The southwest metropolitan Mexico City area has one of the most severe air pollution problems in the world, with a daily ozone concentration of 0.12 ppm or more. Studies of children living in Mexico City showed that they had more DNA damage in nasal epithelium than children living in a port by the Pacific Ocean with a low level of pollution (33). The effect of air pollution in Mexico City was also investigated in young non-smoking adults who moved to the southwest metropolitan Mex-

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ico City area from a Pacific port with a low level of pollution. It was found that within 2 weeks of arrival, the level of DNA damage increased in nasal epithelial cells and remained high during the following 10-week period (34).

The discrepancy between the Czech study and the Mexican study may be explained by the different types of air pollution in the two regions. Respirable particles and hydrocarbon compounds are the most important constituents of the air pollution in the Teplice region in the Czech Republic, whereas the air pollution in Mexico City has a high content of ozone and other directly oxidizing components. A study from Mexico City suggested that ozone and other directly oxidizing components of urbane air pollution are more likely to be detected by the comet assay in nasal epithelial cells and leukocytes than hydrocarbons and respirable particles (25). The southern area of Mexico City has a high ambient concentration of ozone, whereas hydrocarbon compounds and respirable particles are the main components of the air pollution in the northern part of Mexico City. It was found that young adults who lived in the southern part of Mexico City had more DNA damage in nasal epithelial cells and leukocytes than people living in the northern part of the city (25). Although this singles out ozone as a significant contributor to the effect of urban air pollution in directly exposed tissues such as nasal epithelial cells and leukocytes, hydrocarbons and respirable particles could exert an effect in the alveoli and bronchial tree rather than in nasal epithelial cells and leukocytes.

Diet

A number of studies have investigated the effect of nutrients, antioxidants, or a combination of antioxidants on the level of DNA damage as assessed by the comet assay in humans (20, 35–39). No effect of daily supplements of up to 350 mg of vitamin C was shown on DNA damage and resistance to H_2O_2 or ionizing radiation *ex vivo* (20, 36), whereas increased *ex vivo* resistance to H_2O_2 and ionizing radiation was reported 2–4 h after a single dose of 500-2000 mg of vitamin C (35, 40, 41). An 8-week dietary supplementation of vitamin E had no effect on the basal level of DNA damage and *ex vivo* H_2O_2 resistance (41). However, after 20 weeks with a daily mixture of vitamins C and E and β-carotene, the number of endonuclease III-sensitive sites decreased, and *ex vivo* resistance to H_2O_2 increased (36). Administration of individual carotenoids had no effect on DNA damage or FPG or endonuclease III-sensitive sites, although there initially was correlation between the levels of oxidized pyrimidines and some plasma carotenoids, and that a single dose of vitamin C immediately increased H_2O_2 resistance *ex vivo* (38, 40). As compared with a period of carotenoid depletion, carotenoid-rich vegetable products decreased DNA damage, and carrot juice also decreased the number of endonuclease III-sensitive sites. Most of the positive effects of these dietary antioxidants have been associated with *ex vivo* resistance of the lymphocytes. Accordingly, it should be considered whether differences measured by the comet assay induced by antioxidant supplementation actually reflect the resistance to handling of samples *ex vivo*, *e.g.,* oxidation during sample work-up or with respect to frozen storage. Moreover, it cannot be determined whether any change in the actual level of DNA damage is the result of an altered damage rate, *e.g.,* due to improved antioxidant defense or to an altered DNA repair capacity.

Exercise

In general, exercise is regarded as promoting good health and well-being, but excessive exercise is associated with oxidative stress, reflected by higher levels of oxidative DNA damage (8-oxodG) and lipid peroxidation (42–44). It is controversial whether or not the indication of oxidative stress is related to an increased risk of disease. However, the cautionary approach is to register exercise habits and analyze these in biomonitoring studies.

Tice *et al.* (45) were the first to report an effect of exercise by the comet assay. After jogging 5 km, one of three persons had an increased level of DNA damage in the blood sample obtained 5 min after the run (45). Although the result indicates that the level of DNA damage is increased in blood cells after exercise, it must be considered anecdotal, and no record concerning the intensity of the run was reported. A series of studies from Günter Speit's laboratory have addressed various aspects of the effect of exercise on the comet assay. They showed that jogging below the aerobic-anaerobic threshold did not result in an increased level of DNA damage (46). Persons subjected to strenuous exercise above the aerobic threshold did not have an increased level of DNA damage immediately after the exercise (6 min), but the level was increased after 6 h and reached a maximum 24 h after the run (an approximately 3-fold increase). Baseline values were reached after 72 h (46). The higher level of DNA damage in the hours after exercise was interpreted as an effect caused by the DNA repair system, *i.e.,* excisions of damaged nucleotides, although there was no evidence of cytogenetic damage, as assessed by SCE (46). A greater level of DNA damage (up to a 100-fold increase) was observed in well-trained athletes after a triathlon race, and the high level of DNA damage remained for a longer period after the race (47). Curiously, there was no alternation in FPG-sensitive sites, urinary 8-oxodG levels, or MN in the time period of 5 days after the triathlon competition, suggesting that the DNA damage seen by the comet assay is not the result of a direct effect of reactive oxygen species related to exercise-induced oxidative stress (47). This is in good agreement with the observation that multivitamin supplementation had no effect on the exercise-induced DNA damage, although the individuals receiving vitamin E supplementation 14 days before the exercise had less DNA damage after exercise (48). As evidenced by the negative result in the SCE and micronucleus assays, exercise apparently does not result in chromosome damage. The increase in DNA damage has been attributed to tissue damage, activation of neutrophils, and inflammation.

It is worth noting that an effect of exercise by the comet assay has not been reported in studies other than those published by Speit's laboratory, although a higher level of strand breaks in leukocytes after exercise has been reported by fluorometric analysis of DNA unwinding (49). In recent years, reports have often stated that individuals were asked about their level of exercise in the days before the blood samplings. However, these reports do not state whether exercise was associated with a higher level of DNA damage, possibly because people who exercise heavily are not selected for the studies or because moderate exercise is not an important determinant for the basal level of DNA damage. Before the effect of exercise is known in sufficient detail, biomonitoring studies should obtain information about the type and intensity of the exercise and the time since the last bout of exercise. The training status of the test person may also be important. Again, a study from Speit's laboratory (50) has shown that trained men had less net induc-

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tion of DNA damage 24 h after a bout of exhaustive exercise on a treadmill.

Gender

The large majority of published biomonitoring studies consist of individuals of both sexes. A statistical analysis is routinely carried out to evaluate any difference between the sexes in the study population. In general, at present, an effect of gender must be regarded as a matter of controversy. The results from a large cross-sectional study of healthy individuals reported that men had more DNA damage than women, displayed as a wider span of DNA damage in the group of men (23). In contrast to the results of the Italian study (23), we recently found that women had more basal DNA damage than men.[4] Apart from these cross-sectional studies, other somewhat coincidental findings from a Polish study (26) of shoe factory workers reported that men had a higher level of DNA damage than women, whereas a study of aflatoxin-exposed individuals from Gambia reported that women had more DNA damage than men (51). It is possible that study design may offer an explanation for the contradictory results reported. For instance, the Italians used single measurements for each individual, whereas we used repeated measurements. However, a German study using repeated measurements did not detect an effect by gender (14). It is worth noting that analysis of 8-oxodG in leukocytes also has provided inconclusive results of differences in oxidative DNA damage according to gender (52). In contrast, there is strong evidence that women have a higher number of MN than men do (53). Therefore, it seems more likely for women to have the highest level of DNA damage, if there is an effect of gender.

Infection

It might be expected that the comet assay would detect more DNA damage in individuals suffering from infectious diseases. It is necessary to discriminate between random infections such as the common cold or influenza and chronic infections. Curiously, we have only found one study in which severe infections in infants were associated with more DNA damage (54). As we saw for exercise, we have noted that in several biomonitoring studies, data on health status were recorded, but the contribution of health status to the variation in statistical analyses is never provided.

Residential Radon Exposure

Exposure to residential radon was associated with a higher level of DNA damage among family members living in houses with a radioactive radon concentration above 200 Bq/m^3 (55).

Smoking

As seen with the development of other tests for DNA damage, smoking is always one of the first exposure circumstances to which researchers turn their attention as a source of an agent that should produce a positive effect. In addition, biomonitoring studies often include both smokers and nonsmokers. A study of 200 individuals from Italy showed that smoking increased the extent of migration in lymphocytes (10% increase in migration among smokers), but there was no effect detected by SCE or no relationship to the amount of cigarettes smoked per day (16, 17, 23). Individuals who quit smoking had reduced basal levels of DNA damage 1 year after cessation (17). A Greek study consisting of men ages 20–25 years or 55–60 years showed that smoking was associated with an increased level of DNA damage (40%; based on an average of the whole study) for both the young age group and the old age group (32). A number of occupational studies from Turkey (27, 56), France (57), and Poland (58) have also reported an effect of smoking. Recently, a study from China found that both smokers and employees in a cigarette factory had more DNA damage than nonsmoking controls who were not occupationally exposed to tobacco dust (59). A study of Scottish men ages 50–59 years showed that smokers had 1.6-fold more endonuclease III-sensitive sites than nonsmokers, although there were no convincing effects of smoking on the basal level of DNA damage (36). In recent years, an overwhelming amount of publications (usually reports from occupational studies) have failed to show any effect of smoking (9, 19, 22, 24, 28, 29, 55, 60–64). One study has even reported that smokers had less DNA damage than nonsmokers (65). The discrepancy in the reports is not surprising because smokers had only a slightly higher level of DNA damage in the positive studies. Several of the negative studies have been small and have detected the effects of occupational exposure as well. Thus, the failure to show an effect of smoking could be due to low statistical power. However, it is striking that determination of 8-oxodG in leukocytes has produced a similar discrepancy (52). We have noted that the positive studies seem to cluster in the southern part of Europe (Italy, France, Greece, and Turkey; Refs. 16, 17, 23, 27, 32, 56, and 57). It is tempting to speculate that geographical variation in smoking habits may be an underlying factor that can explain the positive results of smoking is some parts of Europe. It is possible that brands of cigarettes containing dark tobacco and high tar content are smoked more frequently in the southern part of Europe. The effect of smoking may also be explained by differences in the composition of the diet in the southern part of Europe, including foods rich in antioxidants or vitamin supplementation.

Sunlight

Seasonal variation has been reported by the comet assay in some laboratories. Longitudinal studies by Betti *et al.* (16) and Frenzilli *et al.* (17) found that more DNA damage was detected in samples obtained during the summer months than in samples obtained during other times of the year. Likewise, unpublished observations from studies carried out by the group of Ray Tice in the United States indicate that more DNA damage was present in samples obtained during the summer than in those collected during the winter.[5] We have also reported a seasonal effect by both the comet assay and UVC-induced unscheduled DNA synthesis, and we were able to correlate the seasonal variation to the exposure of the individuals to sunlight (9). To further characterize the seasonal variation, we set up a large study among healthy individuals, and we have found that exposure to sunlight is the factor that best accounts for the variation.[6]

Use of the Comet in Assessment of DNA-damaging Exposures in Occupational Settings

In the early 1990s, the comet assay was suggested to be a suitable assay for monitoring occupational exposure. Approx-

[4] P. Møller, H. Wallin, E. Holst, and L. E. Knudsen, unpublished observations.

[5] Ray Tice, personal communication.

[6] P. Møller, H. Wallin, E. Holst, and L. E. Knudsen. Sunlight-induced DNA damage in lymphocytes from humans, manuscript in preparation.

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Table 2 Occupational studies by the comet assay

Environment	Exposure	Subjects[a]	Effect ratio[b]	Notes	Ref. no.
Airport personnel	Motor exhaust	34/11	1.2 (1.0–1.3)	SCE negative, less MN than controls	63
Chemical plant	1,3-Butadiene	19/19	1.1 (0.9–1.3)	HPRT and MN negative, CA and SCE positive	65
Cigarette	Tobacco dust	107/41	1.1 (1.0–1.2)		59
Farmers	Pesticides	41/41	1.0 (0.8–1.3)		68
Gasoline station attendants	Motor exhaust (benzene)	12/12	2.0 (1.2–3.8)	No correlation between exposure and effect	28
Hair colorists	Dyes	13/13	0.8 (0.5–1.2)	SCE and urine mutagenecity (Ames test) negative	27
Hospital technicians[c]	Ionizing radiation	30/30	1.1 (1.0–1.2)		66
Lamination[d]	Styrene	9/7	2.9 (0.4–5.8)	More DNA adducts in exposed group, HPRT mutation frequency negative	77
Lamination	Styrene	27/18	2.0 (1.6–2.4)	CA positive, endonuclease III/FPG protocol negative	64
Lamination	Styrene	13/13	3.2 (2.0–5.7)	More DNA adducts in exposed group, HPRT mutation frequency positive	78
Nuclear plant	Ionizing radiation	49/44	2.3 (1.6–3.6)	Endonuclease III/FPG protocol negative	26
Nurses[c]	Antineoplastic agents	30/30	1.3 (1.1–1.4)		61
Operating room personnel[e]	Anesthetic gases	66/41	2.0 (1.8–2.1)		56
Plastic industry	Vinyl chloride	32/10	2.5 (1.6–4.4)		72
Rubber industry	Complex	19/20	1.1 (0.9–1.3)	MN higher in exposed workers, SCE negative	24
Rubber tire	Complex	30/22	2.8 (2.3–3.4)	Endonuclease III/FPG protocol negative	73
Sewage workers	Waste matter	35/30	1.0 (0.7–1.4)		75
Shoe workers	Organic solvents	34/19	1.1 (0.9–1.4)		29
Waste workers	Waste matter	43/47	2.0 (1.4–3.1)	CA positive, SCE negative	22
Wooden furniture workers[f]	Wood dust	41/34	1.3 (1.2–1.4)		58

[a] Exposed/control group.
[b] The effect ratio is was calculated using the *t* distribution (numbers in parentheses are 95% CI).
[c] The DNA damage was scored by arbitrary classification as undamaged, slightly damaged, damaged, and highly damaged cells. We calculated a mean grade of DNA damage for each person by multiplying the number for cells in each class by 1, 2, 3, and 4, respectively.
[d] The median of a subset of cells with highly damaged DNA from laminators was different from that of the control group ($P = 0.02$, Mann-Whitney *U* test).
[e] The DNA damage was scored as undamaged, minimal, and extensive. We calculated the mean arbitrary DNA damage by multiplying the number of cells in each group by 1, 2, and 3, respectively.
[f] The DNA damage was scored by an arbitrary classification in five classes. We calculated a mean grade of DNA damage for each person by multiplying the number for cells in each class by 1, 2, 3, 4, and 5, respectively.

imately half of the studies show a positive effect of exposure to DNA-damaging agents, whereas the other half do not. Table 2 summarizes the occupational studies in which the comet assay has been used as a measurement of effect. For each study, we have calculated an effect ratio and the 95% CI between the exposed and control groups, based on the *t* distribution. There is a statistically significant effect ratio if 1.0 is not included in the 95% CI. Information on the mean and SD has been collected directly from studies that have stated these values; otherwise, we have approximated the values from graphs. In four studies (56, 58, 61, 66), the level of DNA damage was reported as grade or type, and we have calculated a mean arbitrary score on basis of the whole cell population (a detailed description of the calculation of the mean arbitrary score for these four studies is outlined in the footnotes of Table 2).

1,3-Butadiene Exposure

The exposure and biological effects of 1,3-butadiene have been investigated by several assays, including the comet assay, among workers in a 1,3-butadiene production plant. 1,3-Butadiene is monomer compound that is used in the production of synthetic rubber and other polymer products. It has been classified as a compound that is probably carcinogenic to humans (group 2A; Ref. 67). As monitored by personal passive dosimetry, workers in the production unit were exposed to higher levels of 1,3-butadiene compared with the control group from the same plant. The results obtained by the comet assay did not indicate that the exposed workers had more DNA damage than unexposed referents (60, 65). However, by subdividing the data by smoking and exposure status, it was found that 1,3-butadiene was associated with more DNA damage in the smokers. This may suggest an interactive DNA-damaging effect between smoking and 1,3-butadiene. Determination of MN frequency and HPRT mutation frequency gave the same results in the exposed and unexposed group. In contrast, there were increased CA and SCE frequencies among all exposed workers, which showed a relationship to exposure to 1,3-butadiene (65).

Farmers

In a study of French farmers, blood samples were obtained the day before and 1 day after spraying fields with pesticides. There was no statistically significant difference between samples taken before and after spraying (difference in tail moment, $P = 0.26$, Student's *t* test; Ref. 68). It was also reported that samples obtained during the summer had more DNA damage than samples obtained during the winter, and this was suggested to be due to ambient exposure to pesticides during the summer (57). We have suggested that there is an effect of exposure to solar radiation by the comet assay (see "Sunlight"), and we speculate that the seasonal variation might be due to exposure to sunlight.

Hair Colorists

The effect of occupational exposure to hair dyes has been studied among professional hair colorists who had 2–25 years of working experience with hair dye products and did not use gloves during the application of the dyes. There was no difference in the level of DNA damage among the hair colorists and referents, and the results of SCE and urine mutagenicity were negative as well (27).

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Hospital Workers

Two studies have assessed health effects on hospital workers who were exposed to either antineoplastic agents (61) or anesthetic gases (56). In the first study, nurses who worked in the oncology department had more DNA damage than unexposed controls (61). Use of safety protection equipment such as gloves, masks, gowns, and eye glasses was associated with a lower level of DNA damage among exposed nurses. In the second hospital worker study, a higher level of DNA damage was observed among operating room personnel, who were exposed to anesthetic gases (mostly halothane, nitrous oxide, and isoflurane; Ref. 56).

Ionizing Radiation

The effect of chronic occupational exposure to low-dose ionizing radiation has been investigated among hospital technicians in Turkey (66) and among industry workers in Poland (26, 69). The exposed hospital technicians had a trend toward a higher level of DNA damage ($P < 0.10$, Student's t test, based on the whole population of scored cells; Ref. 66). There was a 2-fold higher level of DNA damage among the Polish industry workers, but no difference in endonuclease III- or FPG-sensitive sites was seen between exposed and unexposed workers (69). This was unexpected because both endonuclease III and the FPG protein detect oxidative DNA damage. However, the effect may be due to an adaptive response toward oxidative stress conferred by repeated exposures to ionizing radiation. For instance, it has been reported that leukocytes obtained from children living in the Chernobyl region are protected from (or have a lower responsiveness to) *ex vivo* damage of bleomycin (70). Up-regulation of antioxidant and/or DNA repair enzymes is a possible mechanism behind the protective effect.

Organic Solvents and Hydrocarbons

Three studies have investigated occupational exposure to mixtures of organic solvents and hydrocarbons. The exposed groups included airport personnel (63), gasoline station attendants (28), and shoe factory workers (29). Both the airport personnel and the gasoline attendants were exposed to variety of DNA-damaging agents such as motor vehicle exhausts and gasoline vapors, including benzene. The gasoline station attendants and the airport workers had more DNA damage than the control groups (28, 63). There was not a clear pattern between the DNA damage and the level of exposure in the gasoline station attendant study (28). The airport workers had similar SCE but less MN than the control group (63). This unexpected result was suggested to be due to the type of exposure or to the fact that the control group members were, on average, 13 years younger than the members of the exposed group (63).

The study of shoe factory workers included employees from two shoe factories. In both factories, acetone, gasoline, and toluene were detected in the air of the workplaces, and in one of the factories, ethylacetate and diisocyanate were also detected. There was no difference in the level of DNA damage between exposed workers and controls from the factory. The study did not find any relationship between *GSTM1* polymorphism and the level of DNA damage as detected by the comet assay (29).

Plastic Industry. Vinyl chloride is a potent carcinogen that is activated by cytochrome P-450 enzymes in the liver to chloroethylene oxide, which spontaneously rearranges to chloroacetaldehyde (71). This active metabolite reacts with DNA to form N^7-(2-oxoethyl)guanine and exocyclic ethenoadducts. Vinyl chloride-exposed workers in a plastic production plant had more DNA damage than members of a referent group (72). Air samples taken by personal air samplers of exposed workers detected vinyl chloride levels that were below the permissible value (<5 ppm). The level of increased DNA damage between exposed workers and controls was in the range of 1.9-fold (0.5–2 years of employment) and 3.2-fold (5–10 years of employment; Ref. 72). It was speculated that the association of DNA damage level and duration of employment was due to the accumulation of highly persistent vinyl chloride-generated ethenoadducts. However, if ethenoadducts can be detected by the comet assay, they should be alkaline labile, and to our knowledge, this is not known.

Rubber Industry. A large number of chemicals are used in the production of rubber, including vulcanization agents, colorants, solvent, accelerating agents, and activating agents. Two studies have shown that workers in rubber production plants tended to have more DNA damage than controls. In one of the studies, the level of DNA damage in 30 workers in a rubber tire factory in the Slovak Republic was more than 2-fold higher than that of controls (73). A 1-year follow-up study showed that the rubber tire workers still had more DNA damage than the controls, as well as increased frequency of MN and CA (74). In the second study, lymphocytes obtained from 19 male rubber workers at a plant in northern Italy had slightly more DNA damage than 20 age-matched controls, although the difference was not statistically different at the 5% level (24). The cytogenetic assays revealed that exposed workers had more MN, whereas there was no difference in SCE between the exposed and unexposed groups (24).

Sewage and Waste Disposal Occupations. Studies of workers exposed to waste materials in a waste disposal site (22) and sewage workers (75) have been carried out. Twofold more DNA damage was observed in the waste disposal workers than in the control group (22). There was also an increased frequency of CA, yet there was no correlation between the CA and the comet assay. In contrast, SCE was not increased in waste workers as compared with the control group (22).

The study of sewage workers did not show any effect related to exposure to waste materials (75). The sewage workers were classified into three different groups of exposure, based on self-reported levels of exposure, and the group with the highest exposure was defined as those workers who had worked in sewage-contaminated environments for at least 8 h in the preceding 2 weeks. This may indicate that the sewage workers are not exposed to waste matter to an extent that causes DNA damage in lymphocytes. However, the assessment of exposure may not accurately discriminate between different exposure circumstances. It would have been of great value if some sort of definition of the DNA-damaging agents and measurements of exposure had been included in the study.

Styrene Exposure

Styrene has been classified as a possible carcinogen to humans (group 2B) by the IARC (76). It is used in the production of reinforced plastic fabrications used in boats, corrosion-resistant tanks, pipes, and car parts, and 10% of styrene may evaporate during lamination (76). There have been reports from two studies examining styrene-exposed workers in plastic lamination plants (64, 77, 78). In one study, a higher level of DNA damage and CA was observed among exposed workers (64). More DNA damage was also detected in the lamination workers than in the referent group in the second study (78). A study performed 3 years earlier had indicated an effect, although the

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Table 3 Response of white blood cells in comet assay after administration of therapeutics to cancer patients

Agent	Disease	Subjects[a]	Effect ratio[b]	Interval[c]	Notes	Ref. no.
^{131}I	Thyroid cancer	11	1.1 (0.9–1.5)	≈6 d	No effect 2 h after treatment	21
^{131}I	Thyroid cancer	28	1.0 (0.9–1.2)	7 d	No effect, possibly due to complete repair	89
^{131}I	Thyroid cancer/goiter	22	≈3			90
^{131}I	Hyperthyroidism	16	1.2 (1.0–1.3)	30 d	No effect after 1 week	91
Cyclophosphamide	Breast cancer	17	1.5 (1.1–2.2)[d]	1–21 h	Damage most pronounced 16–21 h after treatment	92
Cyclophosphamide	Vasculitis/collagen disease	11/11	1.7 (1.4–2.2)[e]	Not specified[d]	Higher SCE frequency in the group	93
Cyclophosphamide & cisplatin	Breast cancer	11	3.6 (2.2–7.4)[e]	Not specified[d]		94
Dacarbazine	Malignant melanoma	37	11.4 (4.7–∝)[f]	4 h	Less damage after 24 h	95

[a] One number, own control; two numbers, referent group included.
[b] The effect ratio is was calculated using the *t* distribution (numbers in parentheses are 95% CI).
[c] Time between administration and analyses (d, days; h, hours).
[d] The time interval between cessation of treatment and sampling is not specified.
[e] No relationship between dose and response.
[f] Estimated from graphs.

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difference was not statistically significant at the 5% level (77). The lamination workers also had more styrene-specific (O^6-guanine) adducts detected by ^{32}P postlabeling, and a higher HPRT mutation frequency was seen in the lamination workers compared with unexposed factory workers and a second control group outside the factory [laboratory group (77, 78)]. The general impression from the studies is that styrene-exposed workers had more DNA damage, as determined by the comet assay.

Wood Dust

The IARC has assessed wood dust as being carcinogenic to humans (group 1), based primarily on the observation that occupational exposure to wood dust is associated with cancer in the nasal cavities and paranasal sinuses (79). The results of a Polish study showed that workers employed in a wooden furniture factory had more DNA damage in lymphocytes than a control group (58). The data support a previous study performed by the authors (80) that showed that woodworkers had more DNA damage than controls.

Discussion

The main objectives of this review were to consider which factors may or may not play a role in biomonitoring by the comet assay and to evaluate whether or not the comet assay is really useful as an assay to determine risk of individuals in biomonitoring studies. When using the comet assay in different exposure situations, it is important that the method for the relevant exposure is evaluated and that there is control of important confounders. In the survey, we have taken special care to include reports of possible factors that may confound biomonitoring studies using the comet assay. It does not appear that there is any consensus regarding which factors may be regarded as confounders by the comet assay. Studies from single laboratories indicate that exercise and residential radon exposure are confounding factors, but these confounders need to be investigated in other laboratories also. Effects of air pollution, dietary antioxidants, smoking, and season have been reported by more than one laboratory and should be regarded as confounding factors in at least some countries. The effect of age appears to be of little importance in the comet assay, and, at present, the effect of gender is obscured with reports of both men and women having the highest level of DNA damage. In biomonitoring studies, it is recommendable to record and report the effect of the confounding factors discussed here.

A point of consideration in biomonitoring is the discriminative power of the comet assay *i.e.,* to be truly negative in situations of no exposure or exposures that are not DNA damaging and to be truly positive where an effect of exposure is detected in the comet assay. In four of the negative occupational studies (29, 66, 68, 75), there have been no parallel determinations of genotoxic effect with other assays. In the study of the hair colorists, SCE and urine mutagenicity were negative (27), indicating that it could be a true negative result. There have been mixed results in the remaining negative studies by the comet assay (24, 65, 77). It is striking to observe that on average, there were 47 individuals (median, 46 individuals) enrolled in the negative studies, whereas there were 72 individuals (median, 60 individuals) in the positive studies. This is also reflected by comparing the variation of the control groups; the negative studies had a dispersion coefficient of 0.49 (95% CI, 0.09–0.89), and the positive studies had a dispersion coefficient of 0.33 (95% CI, 0.17–0.48). This clearly suggests that the negative studies often suffer from low statistical power rather than reflecting a situation of no biological effect of exposure. In fact, we have estimated the statistical power in all of the negative studies to be below 30%. The question that obviously emerges is how many individuals should be enrolled in future studies to provide a good chance of detecting a positive result. In an attempt to answer this question, we have calculated the variation in control populations outlined in Table 1, and we have estimated a mean dispersion coefficient of 0.36 (95% CI, 0.27–0.46). This means that if we expect to detect 1.5-fold more DNA damage in an exposed group, it will require that each group consist of 13 individuals (and 20 individuals for the upper 95% CI) to detect a statistically significant difference at a level of 5%. (This is calculated under the assumption that the type II error risk $\beta = 10\%$ and that the SD is the same in the exposed and the referent group).

We have found 11 studies that are positive by the comet assay in various occupational studies. Two of the studies reported positive CA results (22, 64) and two studies reported negative SCE results (22, 63). Eight of the 11 studies had no parallel determination of effect by other assays (26, 28, 56, 58, 59, 61, 72, 73). Consequently, it is difficult to provide a firm conclusion of the positive results by the comet assay in investigations of occupational settings. Furthermore, it disturbs the

general picture of the comet assay as a sensitive test of DNA-damaging effects when a large number of studies have not found an effect of smoking, an exposure circumstance that is otherwise regarded as a positive control for a DNA-damaging exposure. Also, the comet assay did not detect an effect of aflatoxin exposure in a population from Gambia (51). There have been reports from some studies investigating the effect of chemotherapy and radioactive iodine therapy by the comet assay (Table 3). It is reassuring to note that an effect has been observed by the comet assay in these few studies where known DNA-damaging agents are given to patients in the course of treatment. However, the effect rarely exceeded a 2-fold increase above the pretreatment level of DNA damage, and there was no dose-response relationship between the administered chemical and DNA damage. This may indicate that the comet assay should not be used to compare DNA-damaging hazards between exposure levels. A positive result by the comet assay should be regarded as an indication of a biological effect related to some environmental or occupational agent. It is worth stressing that the comet assay seems to be well suited as a fast and inexpensive test of biological effect, but at present, it should not be used in the assessment of individual health risk of humans. This observation is further underlined by our current knowledge that the comet assay is susceptible to confounding factors such as sunlight, exercise, infections, air pollution, and dietary factors, which are not easily assessed.

Three occupational studies showed negative findings by the endonuclease III and FPG enzyme treatment but higher basal levels of DNA damage (26, 64, 73). There have been studies reporting a lower level of endonuclease III- and FPG-sensitive sites after dietary antioxidant supplementation. Also, the usefulness of the enzymes was demonstrated among individuals treated with hyperbaric oxygen pressure (81).

The comet assay is suitable for monitoring levels of DNA damage in cell types other than lymphocytes. In biomonitoring studies, nasal epithelial cells and buccal cells have drawn the most attention because they are cells from tissues that come into direct contact with ingested or inhaled compounds. It has been reported that smokers have higher levels of DNA damage in both nasal epithelial cells and buccals cells (82) and nasal epithelial cells (83). Also, the effects of air pollution have been detected in nasal epithelial cells and buccal cells (25, 33, 34).

In conclusion, the experiences of the comet assay in biomonitoring studies are still relatively new, although the use of the assay has been exploited in many laboratories. An impressive, wide variety of exposures has been tested with success by the comet assay, and this supports our basic notion that the comet assay is an excellent screening test for exposures that may be DNA damaging. Many reports of confounding factors have emerged in the studies. It appears that usage of the comet assay is in a phase where we have identified numerous confounding factors that may or may not be relevant for determination of the basal level of DNA damage. The problem we are facing now is that the effect of a confounding factor usually has been tested in a study designed for the purpose, whereas the factor may have no effect in cross-sectional studies. This makes it difficult to pinpoint one or a few factors that we would recommend should always be incorporated into study designs, *e.g.,* by matched control populations. However, samples from exposed and unexposed populations should be collected at the same time to avoid seasonal variation. Likewise, we recommend that age, gender, and smoking status be used as criteria in the selection of populations. Information on the level of exercise, infection, and diet should be recorded on the day of the sampling.

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