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Review

Single cell gel electrophoresis assay: methodology and applications

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Abstract

The single cell gel electrophoresis or Comet assay is a sensitive, reliable, and rapid method for DNA double- and single-strand breaks, alkali-labile sites and delayed repair site detection, in eukariotic individual cells. Given its overall characteristics, this method has been widely used over the past few years in several different areas. In this paper we review the studies published to date about the principles, the basic methodology with currently used variations. We also explore the applications of this assay in: genotoxicology, clinical area, DNA repair studies, environmental biomonitoring and human monitoring. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Single cell gel electrophoresis; Comet assay; DNA

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1. Introduction

In the last two decades, the search for new methodologies which are able to assess DNA damage have been developed. Rydberg and Johanson [1] were the first to directly quantitate DNA damage in individual cells by lysing and embedding them in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. After neutralization, cells were stained with acridine orange and the extent of DNA damage quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer. To improve the sensitivity for detecting DNA damage in isolated cells, Ostling and Johanson [2] developed a microgel electrophoresis technique, commonly known as the Comet assay. In this technique cells embedded in agarose gel were placed on a microscope slide, the cells lysed by detergents and high salt treatment and the liberated DNA electrophoresed under neutral conditions (pH of 9.5) which means that no separation of DNA strands occurred after electrophoresis of gamma-irradiated cells; the DNA then stained with a fluorescent dye (ethidium bromide), resembled a comet with head and tail. However this technique permits the detection of double-stranded DNA breaks only and the presence of RNA can lead to potential artifacts, due to this utility it been limited to studies involving radiation and radiomimetic chemicals [3–5].

Two versions of the Comet assay are currently in use, one introduced by Singh et al. [6], who used alkaline electrophoresis (pH>13) to analyze DNA damage after treatment with X-rays or H_2O_2, which is capable of detecting DNA single-strand breaks and alkali labile sites in individuals cells. This version is known as the ''single cell gel electrophoresis (SCGE) technique'', although for historical reasons many investigators refer to this method as the ''Comet assay''. Subsequently, Olive and co-workers developed versions of the neutral technique of Ostling and Johanson, which involved lysis in alkali treatment followed by electrophoresis at either neutral [7] or mild alkaline (pH 12.3) conditions [8] to detect single strand breaks.

The Singh and Olive methods are identical in principle and similar in practice, but the Singh method appears to be at least one- or two-orders of magnitude more sensitive [9,10].

In the Singh version of the assay, a single cell suspension of the mammalian cell culture or tissue under study is embedded in low-melting-point agarose in an agar gel sandwich on a microscope slide, lysed by detergents and high salt concentration at pH 10 and then electrophoresed for a short time under alkaline conditions. Lysis removes the cell contents except for the nuclear material. DNA remains highly supercoiled in the presence of a small amount of non-histone protein but when placed in alkali, it starts to unwind from sites of strand breakage. Cells with increased DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a ''comet tail'' (Fig. 1).

Depending on pH conditions for lysis and electrophoresis, the sensitivity of the technique can change. Employing neutral conditions for both variables, allows to detect DNA double strand breaks; but the pH 12.3 detects single strand breaks and delay DNA repair sites, while at pH 13 the sensitivity allows to evaluate alkali labile sites, single strand breaks and delay repair sites of DNA, hereby is important to know the purpose of the study.

About the sensitivity of the (SCGE) Comet assay, McKelvey-Martin et al. [11] and Collins et al. [12] reported that the assay resolves break frequencies up to a few hundred per cell, definitely well beyond the range of fragment size for which conventional electrophoresis is suitable.

The present paper reviews and discuses methodology modifications and applications of the SCGE/Comet assay. For earlier reviews, see McKelvy-Martin et al. [11], Fairbairn et al. [13] and Tice [10,14].

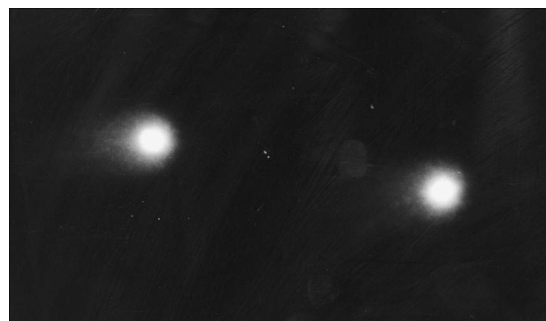

Fig. 1. Photograph showing typical appareance of a ''Comet'' image. Human lymphocytes (amplification 60×).

2. The SCGE/Comet methodology

The basic procedure of the technique is described in detail in various papers [6,11,15]. Briefly, cells are mixed with 0.5% low-melting agarose at 37°C and then placed on a microscope slide coated with 0.5% normal agarose. When the agarose has solidified, an additional layer of agarose is added. After the preparation of the three layers of this material, the cells are lysed in a detergent solution for at least 1 h and then the slides are put into an alkaline or neutral buffer in a electrophoresis chamber, allowing the DNA unwinding, the electrophoresis is carried out, resulting in the migration of small pieces from the core of DNA, toward the electric field. After electrophoresis the slides are rinsed with neutralization buffer or PBS and cells are stained with a fluorochrome dye (Fig. 2).

In the past years, the (SCGE) Comet assay has had several modifications but the underlying principles are based on the neutral or alkaline version. This assay has technical variables which affect the sensitivity, the main ones are: the composition and pH of the lysing solution; the composition and pH of the electrophoretic buffer; and the electrophoretic conditions basically voltage, amperage and unwinding length and running time (Table 1).

2.1. Lysis solution

Neutral and alkaline lysis solutions are used for double and single strand breaks detection, respectively (Table 1). Selection of which method to use should depend on the purpose of the study. Alkaline lysis, which is more frequently cited in the literature, consists of immersing the cells in a high salt solution with detergents at a pH of 10 to >12 for at least 1 h (for a detailed description see Table 1). Some modifications in the composition of this lysis had been reported by McKelvey-Martin et al. [11]. They obtained similar lysis results using or not using *N*-laurylsarcosine in the detergents mix, another is the addition of PK to remove any residual protein such as Rojas et al. [16] reported for buccal epithelial cells. Olive [17] introduced other composition conditions and pH modification (12.3), in this case the solution only had high salt concentration; in the neutral version the concentration is higher than in other protocols, due to the physicochemical characteristics of DNA, for this reason the use of PK is recommended to remove any residual protein.

2.2. Unwinding and electrophoresis buffer

Prior to electrophoresis, the slides are equilibrated in alkaline electrophoretic solution, which contains low salt, no detergents and higher pH (>12.3) generally. The reported time difference during both the pre-electrophoresis wash or unwinding and electrophoresis steps can be attributed largely to the extent of damage and desired detectability. Neutral protocol requires more than 1 h of unwinding time to get free of associated proteins with DNA. Much of the variation in the reported protocols is found during electrophoresis. The desired voltage and time of electrophoresis will obviously be related to the levels of DNA damage expressed in the cells and the salt concentration of the running buffer. Since DNA is required to migrate only a fraction of a millimeter for microscopic observation, significant DNA migration, which leads to comet formation, is possible with very short electrophoresis runs (5–30 min) and low voltages (0.5–5 V/cm) as compared to the most conventional DNA electrophoretic techniques. The length of unwinding (alkaline) and the duration of the electrophoresis are variables which depend on the cell type being investigated and the type of damage being assessed. It is important to consider some changes in these steps, larger comets can be obtained by using a higher voltage or time of electrophoresis. Table 1 shows the most frequently used conditions in neutral, mild alkaline and alkaline assays. Greater sensitivity may be achieved in the assay by increasing the length of time between placing the slide in electrophoresis buffer and applying the current. Green et al. [9] reported that increasing this time beyond 40 min causes an increase in comet formation on control slides. This period of incubation is nominally to allow unwinding of DNA to be initiated from strand breaks but its main function might be to allow the high salt lysis solution to diffuse out of the agar on the slide, where it competes with DNA as an electrolyte. Increasing the temperature of incubation for unwinding and electrophoresis increases the assay sensitivity but also comet formation in controls. A temperature of 15°C appears to give maximum discrimination. This temperature, and the 40

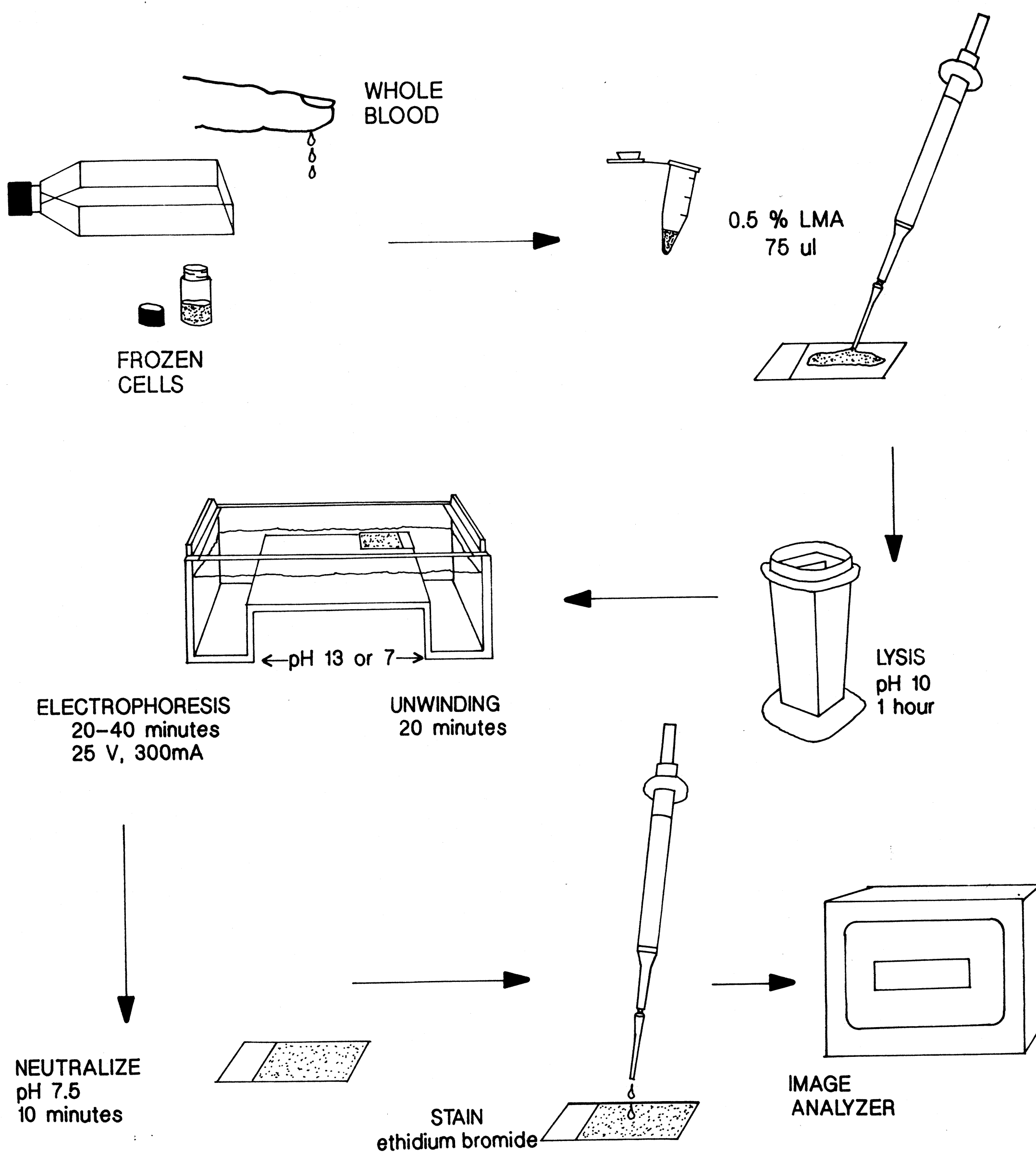

Fig. 2. SCGE basic methodology.

min unwinding time, give maximum sensitivity but are on the borderline of producing acceptable controls. Some researchers might prefer to trade a slight loss of sensitivity in order to obtain a robust assay.

2.3. Other experimental variables

Cell suspension preparation, slides and gel size preparation, time of neutralization, dehydration of

Table 1
Principal SCGE methodology

Technical variables	pH 7	pH 12.3	pH >13	
			Damage	DNA-repair
Number of layers and concentration of agarose	2–3 layers 0.75%	2–3 layers 0.75%	1–3 layers 0.5–1%	2 layers 1%
Lysing buffer	EDTA (0.025–0.03 *M*) SDS (0.5–2.5%) Some authors add 10 mg/ml of (PK) proteinase K >1 h	NaCl (1.0 *M*) NaOH (0.03 *M*) Sometimes add 0.5% of *N*-laurylsarcosine and 2 m*M* EDTA >1 h	EDTA (100 m*M*) NaCl (2.5 *M*) Tris (10 m*M*), pH 10 optional 10 m*M* *N*-laurylsarcosine in fresh add 1% of Triton X-100 when samples containing erythrocytes addition of 10% of DMSO is recommended. Sometimes PK 10 mg/ml is added	EDTA (100 m*M*) NaCl (2.5 *M*) Tris (10 m*M*), pH 10 optional 10 m*M* *N*-laurylsarcosine in fresh add 1% of Triton X-100 when samples contain erythrocytes addition of 10% of DMSO is recomended
Incubation with enzymes	Is not required	Is not required	Is not required	Placed in a trough containing enzyme buffer at 4°C for 5 min. Add 50 μl of buffer for enzyme[a], at 37°C for 5 min, two times, at the end stop the reaction with DMSO at 4°C
Unwinding buffer	Boric acid or acetic acid (90 m*M*) EDTA (2–5 m*M*) Tris (40–117 m*M*) Duration 2–16 h	EDTA (1–2 mM) NaOH (0.03 M) pH 12.3 1 h	EDTA (1 m*M*) NaOH (300 m*M*) pH>13 20–60 min	Is not required
Electrophoresis	0.5–0.57 V cm^{-1} Duration is dependent on the voltage and amperage, but generally is for 25 min	0.5–0.67 V cm^{-1} Duration depends on the protocol and cell type, generally is for 25 min	0.8–1.5 V cm^{-1}, 25 V and 300 mA, duration depends on the protocol and cell type, generally is for 10–60 min	20 V for 24 min in a buffer with 300 m*M* NaOH, 1 m*M* EDTA

[a] NaOH (300 m*M*), EDTA (1 m*M*), DMSO (10%, v/v).

the slides, the DNA-specific dye used for visualization, the magnification used to examine the migrating DNA and the method(s) used for data collection and analysis are variables that need to be considered.

2.3.1. Cell suspension preparation

Since the comet assay is designed to evaluate DNA damage in individual cells, clearly the cells or tissues to be evaluated need to be assayed in a way that allows distinction between the cells. Virtually any eukaryotic cell can be processed for the analysis of DNA damage using this assay.

The existence of various methods for generating single cell suspensions is documented in papers covering a wide range of biological fields. The obvious concern when measuring DNA damage and strand break rejoining in tissues from animal or clinical samples is that the samples should be isolated and processed without allowing additional repair or creating additional strand breaks [18,19]. The most commonly examined human cells are leukocytes and lymphocytes. However, many parameters can affect the response of lymphocytes in the assay in terms of the ability to detect damage. Some authors [11,20] reported considerable intra-individual variability of comet formation using the single cell gel assay; also have been discussed a variety of possible factors that may be responsible for these differences including the blood donor's age, the physical activity of the donor, and whether or not the donor smokes [21–24]. Moreover, cell cycle status might be an additional level of complexity, since

chromatin structure affects the role of DNA during comet formation in both alkaline and neutral assay systems, and chromatin structure changes depending the cell cycle phase [13,25].

2.3.2. Slide preparation

Two basic procedures are in use, the slides with only one layer of agarose and the most common employed with three layers of agarose (''sandwich''). In the single layer procedure, cells are suspended in low-melting-point agarose and placed directly on a fully frosted slide. In the ''sandwich'' procedure the cells are also contained in low-melting-point agarose. The cells are placed on a non-frosted slide with a layer of regular agarose. After that another layer of low-melting-point agarose is added. Then the cells are contained in the middle layer of the ''sandwich'' (Fig. 2). The first layer is used to promote an even and firm attachment of the second and third layers, in the single procedure this first layer is substituted by the frost on the slide, however, the manipulation of the gel needs to be more careful. An important improvement to the slide preparation was introduced by Singh and Khan [26] when they dehydrated the first layer of agarose, incubating at 40–50°C for few minutes, creating permanent slides. In the second layer the cells are contained; the concentration of agarose and the dilution of the cells are important parameters for an efficient analysis. Typically approximately 1000 to 50 000 cells are suspended in 10 μl of PBS or culture medium and are mixed with 75 μl of low-melting-point agarose at a final concentration 0.5–1% at 35–45°C (the amount of agarose can be changed in relationship to the gel size). Then if more cells are used the analysis of the comet image could be difficult. The third layer is added only for the protection of the cells (Fig. 3). The concentration and amount of the agarose is an important parameter, which can contribute to the background intensity during microscopic analysis. Some modifications have been done to this procedure. Some authors utilized coverslips instead of slides. Collins et al. [27] introduced an enzymatic procedure to the assay. In this approach the penetration and function of the enzyme is better if the slide has only two layers. In the slide preparation, the major considerations are directed to obtain sufficiently stable gels for subsequent manipulations, as well as guarantee that during comet analysis, the frosting of the slide does not contribute to noise fluorescense which may obscure details of the comets.

2.3.3. Neutralization time

After electrophoresis, the slides are neutralized with Tris buffer, pH 7.5. The original protocol [6] suggests three washes for 5 min each. Supported by our personal observations, when the time of neutralization is extended, the background intensity in the slide decreases.

2.3.4. Permanent slides

The first step already mentioned in Section 2.3.2 is necessary to obtain permanent slides. After neutralization, the layers of agarose are dehydrated immersing the slides twice in absolute ethanol for 5 min each, this modification only has been reported by Singh et al. [28]. With the same purpose Speit (pers. commun.) washes the gel with distillate water for 2 min, then drips out the excess, and dehydrates it in a warm heater until dried.

2.3.5. DNA-specific dye and magnification for data collection

The last step of the technique concerns the DNA-specific dye used for visualization, variables such as fluorochrome used and the method(s) for data collection, largely depend on the investigator's specific needs and presumably have little effect on the assay sensitivity and resolving power. Objective magnification has been used from 160× to 400×, but usually the 200–250× range is used. The magnification that is most appropriate depends on the type of cell being evaluated, the range of migration and the constrains of the microscope and/or the imaging system used. Similarly, selection of a fluorescent dye depends to a large extent on the limitations of the equipment and the manner in which data will be collected. The dyes more frequently used are ethidium bromide, propidium iodide and DAPI. Recently, Singh et al. [29] have reported that the use of YOYO-1 and silver green increases the sensitivity. The results obtained with the scaled objective are limited, because it can measure the distance that DNA migrates from the core, although it fails to detect the amount of DNA in the tail. For this reason, in past years the use of an

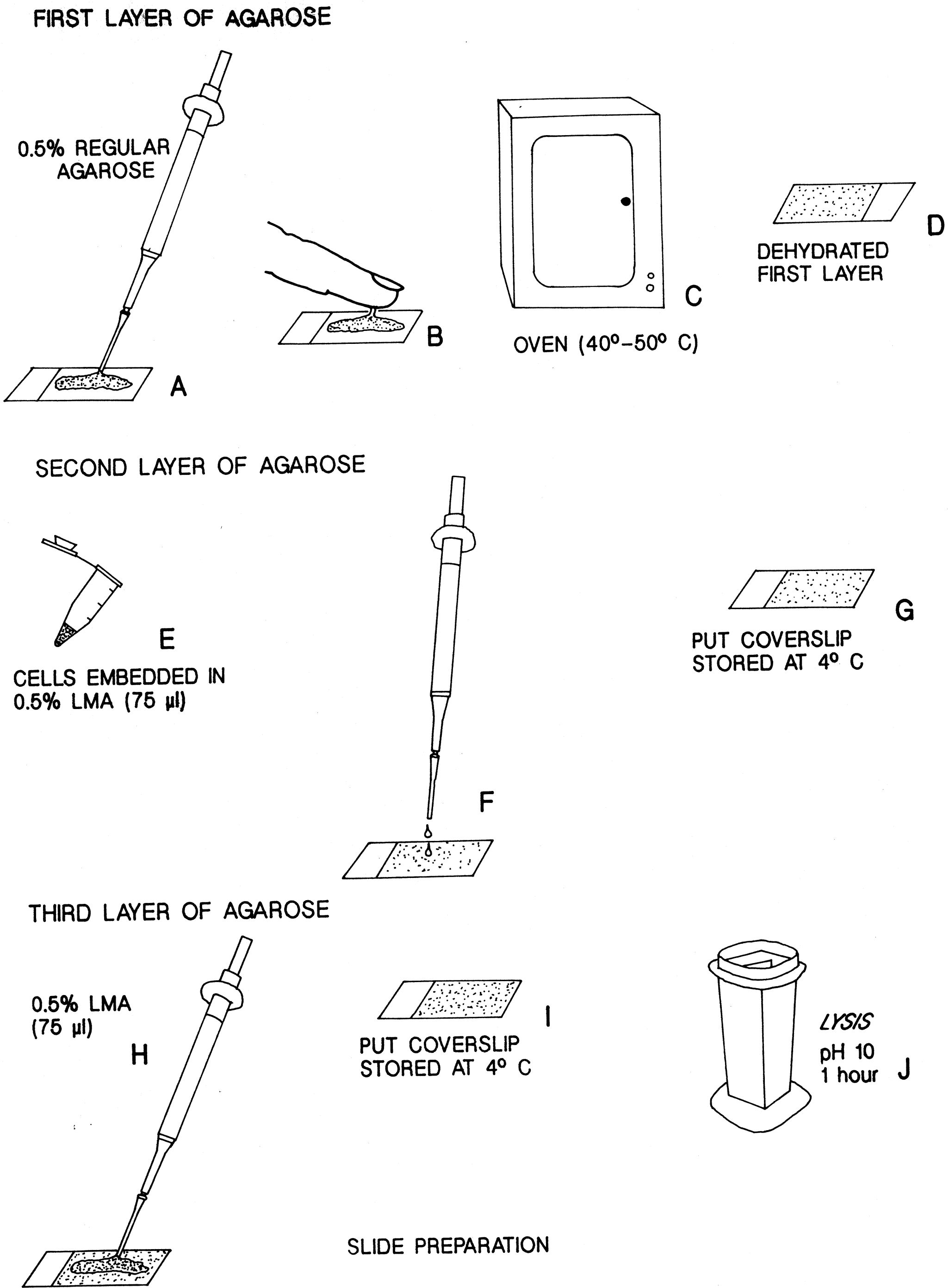

Fig. 3. Protocol for permanent slide preparation.

image analyzer has become generalized. Some authors also used the laser scanning microscope to measure differences in DNA damage in samples subjected to the (SCGE) Comet assay [30].

2.3.6. Data analysis

There are almost as many methods for quantifying DNA damage by this assay as there are scientists using the technique, the most flexible approach for collecting SCGE/Comet assay data involves the application of image analysis technique to individual cells, and several commercially available software programs have been developed specifically for collecting such data. However, methods that do not rely on image analysis can be just as useful. The simplest method for collecting SCGE data is based on determining the proportion of cells with damage (i.e., those exhibiting migration versus those without it, [31]), however, this approach fails to provide information about the extent of the damage among damaged cells. Another approach subclassifies damaged cells into the ones having various degrees of DNA migration as reported by Anderson et al. [32]. The parameter commonly used is the length of DNA migration, usually presented in μm. Migration length is related directly to fragment size, and would be expected to be proportional to the extent of DNA damage. The migration length could be measured using different approaches; with a micrometer in the microscope eyepiece, a rule on photographic negatives/positives of cell images or in the camera monitor, and by using the image analyzer. Currently, the criteria used to identify the trailing and leading edge of the migrating DNA seems to depend on the investigator and/or software program. Furthermore, some investigators use the term ''DNA migration'' to describe total image length while others apply the term to migrated DNA only. A variant of this parameter is to present the ratio of length/width [10,33] or width/length [34], with cells exhibiting no damage having a ratio of approximately 1. Olive and Durand [35] discounted the utility of DNA migration as a parameter for DNA damage in the neutral or pH 12.3 alkaline assay, based on the observation that the length of DNA migration reached a plateau while the percentage of migrated DNA continued to increase. However, this limitation in migration length is not a characteristic of the pH>13 alkaline assay, where length has been reported to be the best parameter for this version of the assay. The computerized image analysis system to collect SCGE/Comet data, favors the evaluation of relative amount of migrated DNA, presented either as the percentage of migrated DNA or as the ratio of DNA in the tail to DNA in the head. This parameter assumes signal linearity in quantifying the amount of DNA ranging over multiple orders of magnitude and that the staining efficiency of the fluorescent dye is identical for migrated and non-migrated DNA [36]. The concept of tail moment (tail length×tail intensity or percentage migrated DNA) as a parameter for DNA migration was introduced by Olive et al. [8]. However, a consensus among investigators as to the most appropriate manner in which to calculate tail moment has not been obtained. Some agents induce long, thin tails while others induce short, thick tails. Such information may provide insight into agent-specific differences in the intragenomic distribution of DNA damage within a cell.

2.3.7. Modifications for DNA repair studies and cross-linking agents

Only a small proportion of DNA-damaging agents (ionizing radiation), induce direct breakage of the DNA phosphodiester backbone. Most agents induce damage to DNA bases. With such agents, strand breakage occurs as an intermediate stage in the repair of the damage. The Comet assay can detect both types of strand breakage and by means of a time course experiment it is possible to distinguish between them. By combining the assay with the use of a purified DNA repair enzyme, the sensitivity and specificity of the assay is greatly increased allowing to detect the specific type of DNA damage [9,37]. The comet assay can detect as few as 200 DNA strand breaks per cell, perhaps down to 50 breaks per cell with modified procedures [9]. The method can be used with almost any cell type, the main restriction being not to induce DNA damage during the preparation of the single-cell suspension. It does not require the use of growing cells, and can be used with material treated in vitro or in vivo.

Some authors [9,37] only use the first two layers of agarose, because these conditions allow better interaction between the cells and the enzymes (fpg and Endo III). After cells lysis, the enzyme is added

to the gel in 50 μl of appropriate buffer, covered with a coverslip, and incubated at 37°C for the required time [12]. Endo III, fpg and uvrA, uvrB and uvrC are the most employed enzymes. Endo III recognizes oxidized pyrimidines, fpg recognizing 8-OH gua and another damaged purines and uvr ABC for bulky lesions and cleavages the DNA backbone during their removal. The next step is to run the electrophoresis (without unwinding) and rinse the slides dropwise with neutralizing buffer and then continue with the classical protocol.

3. Applications of the assay

3.1. Studies on genotoxicity

An useful application of the alkaline version of the (SCGE) Comet assay is in the area of genetic toxicology and a number of investigators have used this version to evaluate in vitro and/or in vivo genotoxicity of several chemicals. A variety of normal and transformed cells including human, animal and plant have been used for in vitro studies.

Most of the work with human cells, have used leukocytes and lymphocytes but other tissues have been also used such as epithelial cells (lens, buccal, nasal and gastric mucosa, skin-derived and foreskin-derived cells), reproductive cells, colon cells, neonatal fibroblast, pancreatic cells, adenocarcinoma cells, lymphoid cells. Different human cell lines have been used including cells from blood (Raji, TK6, HUT-78 and HL-60, MOLT-4), different kinds of carcinomas (cervix [SiHa, HeLa], colon [WiDr, HT-29], bladder [A1698], prostate [Du-145]), melanoma (MeWo, HT-144), glioma (U-87), fibroblast (IBR-3), breast keratinocyte (NHEK-267-1), lung cells (MRC5CV1), etc.

A great amount of work in this field has been done using animal cells, since only a few cells are required for analysis, virtually any tissue or organ is available for investigation. Rodents are the more frequently used but other animals such as dog, sheep, tadpoles and fish have been also employed. This technique has been applied to several organs and tissues of this animals, including blood, bone marrow, brain, gastrointestinal mucosa, kidney, liver, lung, nasal mucosa, ovaries, skin, spleen and testis. The only requirement is that a sufficient number of single cells is obtained for analysis without induction of damage. There are different approaches to obtain the cell suspension, as the use of collagenase and trypsin [38] or just mince the tissue in cold buffer [39], to obtain a nucleus suspension a chilled buffer with NaCl (0.075 *M*) and EDTA (0.24 *M*) at pH of 7.5 is used [40]. Depending on the intrinsic organ conditions, it is possible to improve the isolation additioning specific chemicals as *N-tert*.-butyl-α-phenylnitrone a spin trapper used on brain cells [28], for the better cell preservation. Some groups use the same conditions for all the organs tested, but the optimal conditions for each organ seems to be different, then it depends on the focus of the work (a panoramic evaluation of genotoxicity comparing the DNA damage in different organs or a specific tissue study) the standardization of the conditions for each work are required. Animal cell lines, usually arising from different rodent cell types, the most used are CHO cells HIT-T15, V-79, L5178Y, SCCVII, and primary cultures of hepatocytes had mainly been reported. An interesting aspect of the rodent hepatocyte is the ability to measure DNA damage in parenchymal and non-parenchymal cells simultaneously [41], this ability permits a simple method for discriminating between direct-acting genotoxicants and those requiring metabolic activation [10]. The results are particularly appealing because they include information about the distribution of damage in the target tissue [13]. This distribution among cells provides information on the relative heterogeneity of the damage among cells in different tissues and indicates the proportion of damaged to undamaged cells for each dose and tissue [10], an advantage that no other system can offer. Recently some groups adapted this assay to plant tissues like *Allium cepa* [273].

The amount of chemicals tested in this area is rapidly increasing every year, the first practical use of the (SCGE) Comet assay in the genotoxicity testing was an evaluation of the mutagenic potencies of agents generated by the treatment of potassium perganmanate with acidic solutions [42], from this first report to the present day a great amount of chemicals have been tested, such as metals, pesticides, opiates, pesticides, nitrosamines and antineoplastic drugs (for a more detailed list see Table 2).

Table 2
Genotoxicity studies

Agent	Cell type	Result	Refs.
p-Aminoazobenzene	Liver, lung, spleen, kidney, bone marrow from mouse	+	[95]
p-Benzoquinone	Human lymphocytes	−	[68]
p-Dichlorobenzene	Liver, lung, spleen, kidney, bone marrow from mouse	+	[95]
β-Estradiol	Human sperm and peripheral lymphocytes	+	[128,129]
p-Nitrophenol	V79 cells, human white blood cells	−	[126]
β-Propiolactone	Mouse skin keratinocytes	+	[106]
1,1,2-Trichloroethane with and without S9 mix	Human lymphocytes	+	[116]
1,1,3-Trichloropropane with and without S9 mix	Human lymphocytes	+	[116]
1,2,3,4-Diepoxibutane	Bone marrow and testicular cells from CD-1 mice and Sprague–Dawley rat	+	[125]
1,2,3-Trichloropropane with and without S9 mix	Human lymphocytes	+	[116]
1,2-Dichloroetylene with and without S9 mix	Human lymphocytes	+	[116]
1,3-Dichloropropane with and without S9 mix	Human lymphocytes	+	[116]
1,3-Butadiene	Bone marrow and testicular cells fron CD-1 mice and Sprague–Dawley rat	−	[125]
2,4-Diaminotolueno	Liver, lung, spleen, kidney, bone marrow from mouse	+	[95]
2-AF	Liver, lung, spleen, kidney, bone marrow from mouse	+	[100]
2-Nitropropane	Bone marrow from Wistar rat	+	[132]
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)	HL-60 cells, liver, kidney, lung, brain and mucosa samples from mice	+	[137,138]
4NQO	Testis fron Wistar rat, CHO cells, TK-6 cells	+	[104]
5-FU	Liver, lung, spleen, kidney, bone marrow from mouse	−	[100]
Acetaldehyde	Human lymphocytes	+	[26]
Acetochlor	Nasal cells from rat	−	[89]
AHH	Peripheral blood from BALB/C mice, peripheral blood from C57BL/6 mice	+	[131]
Alachlor	Human lymphocytes	+	[90]
Alkali lysis, pH 12.5	kidney, epididimal sperm of male BALB/C mice	+	[83]
Alkali lysis, pH 12.5	thymocytes, splenocytes of male BALB/C mice	−	[83]
Aloe-emodine	Mouse lymphoma L5178Y	+	[117]
Aluminium	Human leukocytes, human lymphocytes	+	[109]
Aniline derivatives	B6C3F1 mice	+	[136]
Antioxidants	Caco-2, HepG2, HeLa, cells and normal human lymphocytes	−	[121]
Arsenic	Leukocytes and transformed Fibroblasts with sv40, human leukocytes, human lymphocytes	+	[109,110]
Artificial tanning lamps	Human fibroblasts	+	[143]
Atrazine	Human lymphocytes	+	[90]
Auramine	Liver, lung, spleen, kidney, bone marrow from mouse	+	[95]

Table 2. Continued

Agent	Cell type	Result	Refs.
AZQ	BZQR cells	−	[151]
AZQ	K562 cells	+	[151]
BAYy3118	V79 cells	+	[149]
Benzene	Peripheral blood, liver from BDF1-mice, whole blood and bone marrow from NMRI mice	+	[19,93]
Benzene	Bone marrow, spleen from BDF1-mice	−	[19]
Benzenethiol	Human lymphocytes	+	[68]
Benzo(*a*)pyrene	Primary cultures of rat and human hepatocytes, liver from C57BL6 mice, MRC5CV1 cells transformed with sv40, liver, lung, spleen, kidney, bone marrow, stomach, bladder, brain from mouse, peripheral blood from mice	+	[74,78,98] [99–101,133]
Benzo(*a*)pyrene	Lymphocytes and bone marrow from C57BL6 mice, MRC5CV1 cells transformed with sv40,	−	[98,99,102]
Benzo(*a*)pyrene and S9 mix and antiBPDE	MRC5CV1 cells	−	[102]
Benzo-spaced psoralen	CHO cells, AS52 cells	+	[141]
BHC	Liver, lung, spleen, kidney, bone marrow from mouse	−	[95]
Bleomycin	Human lymphocytes, whole blood cells, CHO cells, L5178Y cells	+	[59,60] [66,84]
BZQ	BZQR cells, K562 cells	−	[151]
Cadmium	Leukocytes and transformed fibroblasts with sv40	+	[110]
Caffeine	HL-60 cells	+	[142]
Caffeine and radiation	Embryo cells	+	[115]
Carbendazim	Human lymphocytes	−	[120]
Carbendazim and chlorothalonil	Human lymphocytes	+	[120]
Catalase	Human lymphocytes	+	[66]
Catalase	HIT-T15 cells, human lymphocytes	−	[85,86]
Catechol	Human lymphocytes	+	[68]
CH	TK6 cells	−	[104]
Chlorobenzene	Lymphocytes, from C57BL/6 mice	+	[76]
Chlorobenzene	Bone marrow fron C57BL/6 mice	−	[76]
Chlorothalonil	Human lymphocytes	+	[120]
Ciprofloxacin	V79 cells	+	[149]
Cobalt	Human leukocytes	+	[123]
Colchicine	Liver, lung, spleen, kidney, bone marrow, peripheral blood from mouse	−	[100]
Cyclophosphamide	Whole blood, hepatocyes, liver, testis from mice, human lymphocytes, lymphocytes, bone marrow from C57BL/6 mice, human hepatocytes	+	[3,75,76,133] [77–79]
D-Menthol	V79 cells, human white blood cells	−	[126]
DAB	Liver from mice, peripheral blood from BALB/C mice, peripheral blood from C57BL/6 mice	+	[130,131]
DAB	Lung, kidney, spleen and bone marrow from mice	−	[130]
Danthron	Mouse lymphoma L5178Y	+	[117]
DBCP	Spermatids, spermatocytes, Sertolli cells	+	[91]
DBCP	Testis from Wistar rat	−	[52]
Deoxyuridine	HeLa cells, human lymphocytes	+	[147]
Diadzein	Human sperm and peripheral lymphocytes	+	[128,129]
Diethylstilbestrol	Human sperm and peripheral lymphocytes	+	[128]
Dimethylnitrosamine	Cultured hepatocytes from Alpk:APfSD rat	+	[97]
Dimetridazole (DZ)	Human lymphocytes	+	[86]

(Continued overleaf)

Table 2. Continued

Agent	Cell type	Result	Refs.
DMBA	Peripheral blood and skin tumor cells from mice	+	[79,145]
DMH	Rat colon cells	+	[118]
DMSO	Cultured hepatocytes from Alpk:APfSD rat	−	[97]
Doxorubicin	Human lymphocytes	+	[124]
DZ and 8HQ	Human lymphocytes	+	[86]
DZ and catalase	Human lymphocytes	+	[86]
DZ and SOD	Human lymphocytes	+	[86]
DZ and vit C	Human lymphocytes	+	[86]
Electromagnetic radiation, 2450 Mhz	C3H 10T fibroblasts, U87MG human glioma cells	−	[135]
Electromagnetic radiation, 835.62 Mhz	C3H 10T fibroblasts, U87MG human glioma cells	−	[136]
ENNG	CHO cells	+	[60]
ENU	Liver, lung, kidney, spleen and bone marrow from mice, TK-6 cells	+	[130,104]
Epichlorohydrin	Human diploid fibroblasts (VH-10 cells)	+	[127]
Ethanol	Human lymphocytes	−	[26]
Ethanol	Brain cells of Sprague–Dawley rats	+	[28]
Ethylmethane sulfonate	Primary cultures of rat and human hepatocytes, human lymphocytes, liver, lung, spleen, kidney, bone marrow from mouse, single renal proximal tubular cells, CHO cells, TK-6 cells, AS52 cells	+	[54,60,78] [100,103,104] [105]
Etoposide	V79 cells, human lymphocytes	+	[50,95]
ETU	Liver, lung, spleen, kidney, bone marrow from mouse	+	[95]
Ethylene oxide	Human diploid fibroblasts cells VH10	+	[92]
Fecal water	Caco-2 cells	+	[144]
Flavonoids	Caco-2, HepG2, HeLa, cells and normal human lymphocytes	−	[121]
Fleroxacin	V79 cells	+	[149]
Fluoroquinolones	Lymphoma mice cells L5178	+	[113]
Folate-deficient medium	HeLa cells, human lymphocytes	+	[147]
Genestryl	Human sperm and peripheral lymphocytes	+	[128]
Glutathione	Human lymphocytes	+	[154]
Glutathione and catalase	Human lymphocytes	−	[154]
Glutathione and SOD	Human lymphocytes	+	[154]
Glutathione and UVa	Human lymphocytes	+	[154]
GSM (microwaves)	Human whole blood	+	[146]
GSM and MMC	Human whole blood	+	[146]
GSNO	HIT-T15 cells	+	[85]
H_2O_2	Human lymphocytes, monocytes, human neonatal fibroblasts, peripheral whole blood, hepatocyes, liver, testis, sperm from mice, HeLa cells, hepatocytes from male Fisher 344 rats, V79 cells, traqueal and mesothelial cells from rat, cryopreserved lymphocytes, HepG2, Caco-2, HeLa, GM1899A cells, Raji cells	+	[3,6,43] [50,56,62] [63–65] [66–68] [69–71] [72–74]
H_2O_2+catalase	Human lymphocytes	−	[66]
H_2O_2 and 8HQ	Human lymphocytes	+	[86]
H_2O_2 and catalase	Human lymphocytes	+	[86]

Table 2. Continued

Agent	Cell type	Result	Refs.
H_2O_2 and myricetin	Human lymphocytes	−	[74]
H_2O_2 and quercetin	Human lymphocytes	−	[74]
H_2O_2 and SOD	Human lymphocytes	+	[86]
H_2O_2 and vit C	Human lymphocytes, Raji lymphoblastoid cells	−	[73,88]
H_2O_2 and vit C and vit E	Raji lymphoblastoid cells	−	[88]
H_2O_2 and vit E	Raji lymphoblastoid cells	−	[88]
Hydroquinone	Human lymphocytes	−	[68]
INO2	CHO cells	+	[134]
IQ	Stomach, liver, kidney, lung, brain from mice	+	[101]
IQ	Bladder, bone marrow from mice	−	[101]
Lindane	Human nasal and gastric mucosa	+	[80]
Linuron	Liver from rat	+	[139]
Linuron	Testis from rat	−	[139]
Lithotripter shock waves	CHO cells	−	[111]
Lomefloxacin	V79 cells	+	[149]
m-Phenylenediamine and 2-aminofluorene with and without TX1MX	Human lymphocytes	+	[96]
Maleic hydrazide	Human lymphocytes	+	[90]
Manganese-chloride and permanganate of potassium	Human lymphocytes	+	[42]
Mechlorethamine	TK6 human B lymphocytes cells	−	[47]
MeIQ	Stomach, liver, kidney, lung, brain from mice	+	[101]
MeIQ	Bone marrow from mice	−	[101]
MeIQx	Stomach, liver, kidney, lung, brain from mice	+	[101]
MeIQx	Bone marrow from mice	−	[101]
Metronidazol (MZ)	Human lymphocytes	+	[86]
MMC	Peripheral blood from mice, L5178Y cells	+	[59,79]
MMS	Liver, lung, spleen, kidney, bone marrow from mouse, CHO cells, TK-6 cells	+	[60,100,104,133]
MNNG	V79 cells, mouse skin keratinocytes, liver, lung, spleen, kidney, bone marrow from mouse	+	[50,100,106]
MNU	CHO cells, L5178Y cells	+	[59,60]
MZ and 8HQ	Human lymphocytes	+	[86]
MZ and catalase	Human lymphocytes	+	[86]
MZ and SOD	Human lymphocytes	+	[86]
MZ and vit C	Human lymphocytes	+	[86]
Nalidixic acid	V79 cells	+	[149]
Nonylphenyl	Human sperm and peripheral lymphocytes	+	[128]
Norfloxacin	V79 cells	+	[149]
NSAID	src-Transformed chicken embryo fibroblasts	+	[148]
OPP	Stomach, liver, bladder, kidney, lung, brain, bone marrow from mice	+	[101]
Oxygen hyperbaric	Human leukocytes	+	[152]
Paraquat	Human lymphocytes	+	[90]
PCBs	Human lymphocytes	−	[114]
Phenobarbital	Primary cultures of rat and human hepatocytes	−	[78]
Phenobarbital	Liver, kidney, spleen, lung, bone marrow from mice	+	[95]
PhIP	Liver, kidney, brain from mice	+	[101]
PhIP	Bone marrow from mice	−	[101]
Potassium cyanide	TK6 cells	+	[104]
Praziquantel	V79 cells, human lymphocytes	+	[153]
Propylene oxide	Human diploid fibroblasts (VH-10 cells)	+	[127]

(Continued overleaf)

Table 2. Continued

Agent	Cell type	Result	Refs.
Pyrimethamine	Liver, kidney, lung, spleen and bone marrow from rat	+	[140]
Pyrimethamine	Spleen from mice	+	[140]
Pyrimethamine	Liver, kidney, lung and bone marrow from mice	−	[140]
Radiation	Human lymphocytes, human neonatal fibroblasts, human sperm, sperm, bone marrow fron mice, CHO, CHO-K1 cells, TK6 human B lymphocytes cells, V79 cells, testis from Wistar rats, frozen chicken, meat pork and fish, cryopreserved lymphocytes, human bone marrow mononuclear cells, L5178Y cells, Raji cells	+	[4,6,22] [43–46] [47–50] [51–54] [55–58] [59–61]
Roussin's black salt	HIT-T15 cells	+	[85]
Salted, sun dried and deep-fried fish	Hepatocytes and lymphocytes from fish and mutton	+	[81]
SIN-1	HIT-T15 cells	+	[85]
SIN-1 and catalase	HIT-T15 cells	+	[85]
SIN-1 and SOD	HIT-T15 cells	+	[85]
SOD	Human lymphocytes	+	[66]
SOD	HIT-T15 cells, human lymphocytes	−	[85,86]
Sodium ascorbate	Human lymphocytes, human neonatal fibroblasts, Molt-4 cells	+	[119]
Sodium *N*-lauryl sarcosine	V79 cells, human white blood cells	−	[126]
Sodium-lauryl-sulphate	TK6 cells	+	[104]
Streptocin and steptozotocin	C57BL liver, C57BL kidney, and Lac L Transgenic cells from mice	+	[82]
Styrene oxide	Human lymphocytes, liver, lung, kidney, spleen, bone marrow from mice	+	[94,95]
TBZ	Stomach, liver, bladder, kidney, lung, brain, bone marrow from mice	+	[101]
TGFβ	RPE cells, PVR cells	+	[150]
Trp-P-1	Stomach, liver, lung from mice	+	[101]
Trp-P-1	Bone marrow from mice	−	[101]
Trp-P-2	Stomach, liver, kidney, lung, brain, from mice	+	[101]
Trp-P-2	Bladder, bone marrow from mice	−	[101]
Trypsin	TK6 cells	−	[104]
Turkish herbal extracts	Human lymphocytes	+	[112]
Ultrasound shock waves	CHO cells	+	[111]
Vanadium pentoxide	Testis from mice, human lymphocytes, human leukocytes	+	[107–109]
Vit C	Human lymphocytes, human nucleated cells of whole blood	+	[66,87]
Vit C	Human lymphocytes	−	[86]
Vit E	Human lymphocytes	−	[66]
WC–Co	Human lymphocytes, human leukocytes	+	[122,123]
X-rays and vit C	Raji lymphoblastoid cells	−	[88]
X-rays and vit E	Raji lymphoblastoid cells	−	[88]
Zinc	Human leukocytes, human lymphocytes	+	[109]

The sensibility showed by this assay allow us the use of a potent tool for the study of genotoxicity, approximately 85% from the studies realized in this area found a positive result. However more studies are required to investigate the persistence and repair of the damage observed, the correlation with other genotoxic endpoints as sister chromatid exchanges, chromosomal aberrations, mutations or the induction

of micronuclei, and the most important point, which is the biological meaning of the DNA damage observed by the technique. At this respect Klaude et al. [5] concludes that the use of neutral versus alkaline versions is apparently affecting the behavior of DNA in quite different ways. After neutral conditions the comet tail consists of relaxed loops, whereas alkaline tails are made up from DNA fragments and Collins et al. [12] and Speit et al. [152] comment that the single strand breaks observed in the alkaline version are not the most interesting of DNA lesions because they are quickly repaired and are not regarded as a significantly lethal or mutagenic lesion. However Wagner et al. [105] considers that under specific conditions of sampling, there are good correlation's between the induction of comet tail length, the increase of coefficient of variation using the flow cytometer and the induction of GPT mutations.

3.2. Clinical applications

The possible utilization of this assay in the clinical area was pointed out by Ostling et al. [155] who applied the neutral version to evaluate levels of DNA damage in tumor cells from patients receiving radiotherapy for Hodgkin's disease, non-Hodgkin's lymphoma, squamous cell carcinoma or adenocarcinoma. Due to the few number of cells needed, an analysis can be conducted on the amount of blood obtained by fingerprick or in solid tissues using a fine-needle biopsy technique giving the (SCGE) Comet assay an enormous number of possible applications. Only some of them are mentioned: Olive et al. [156] used the pH 12.3 assay to monitor number of hypoxic cells in irradiated human breast cancer, based on their relative insensitivity to 5–10 Gy X-rays. Differences in the radiation response of tumors of the same type, size and grade has generally been attributed to tumor cell heterogeneity and in particular to differences in intrinsic radiosensitivity, tumor growth kinetics and the presence of resistant subpopulations such as hypoxic cells, at this respect, the (SCGE) Comet assay can provide information on a number of properties of the tumor cells which are known to influence their response such as inherent sensitivity to a particular treatment, proportion of actively growing cells and the presence of lacking oxygen [13,157–160].

Klieman and Spector [161] used the alkaline version to determine the DNA damage in lens epithelial cells sampled from individuals affected with cataracts and from unaffected controls. In addition, studies have been conducted in bladder epithelial cells obtained from the urine of bladder cancer patients [162] and in nasal and gastric mucosa obtained from biopsy material [18].

This assay has been propose to predict the response to chemotherapy, Tice et al. [163] evaluated the DNA damage in cryopreserved peripheral blood lymphocytes from 11 breast cancer patients treated with high doses of cyclophosphamide and cisplatin and given autologous bone marrow transplantation after treatment. Chemotherapy resulted in a significant but variable increase in DNA damage in cell from all patients. Vaghef et al. [164] also reported a variable increase in DNA damage after chemotherapy with cyclophosphamide, 5-fluorouracil and epirubicin in breast cancer patients. Whether the extent of DNA damage correlates with the outcome of treatment remains to be established. The (SCGE) Comet assay may have a future role in the study of the mechanism of action of new drugs and also in the analysis of interactions between antineoplastic agents acting at DNA level. In this respect Kasamatsu et al. [84] reported that the mechanisms involved in the induction of DNA damage leading to strand breakage may vary according to the agent employed. There are agents that induce DNA damage without metabolic activation (direct agents), some that require metabolic activation to induce DNA damage, and others that induce DNA breakage by affecting cellular functions.

Another potentially interesting finding was that the basal level of DNA damage in lymphocytes from patients with different classes of cancer and without any treatment was higher than DNA damage in lymphocytes from people without cancer. The comparatively high level of DNA damage among cancer patients may indicate that the malignant disease is itself associated with increased DNA damage or that these patients have a more fragile DNA than healthy individuals [164,165]. The fact that (SCGE) Comet data can be obtained within a few hours of sampling suggests that this method may be used to monitor

levels of damage in individual patients associated with a regimen treatment and that the regimen could be changed accordingly [10]. For a more detailed list of the works done in this area see Table 3.

3.3. DNA repair studies

Because of its characteristics, the (SCGE) Comet assay has been used to evaluate the ability of virtually any type of eukaryotic cell to repair different kinds of DNA damage, including double- and single-strand breaks and base damage. The neutral and alkaline version of the assay have been used to assess the repair of double-strand and single-strand breaks, respectively. Single- and double-strand breaks induced by ionizing radiation are efficiently repaired in normal cells [4,6,8,22,29,240] (for more detailed data see Table 4). Fifty percent of the damage is repaired within 15 min and complete repair occurs within 1–2 h. This rate of repair has also been demonstrated for subsets of irradiated human lymphocytes [4].

To evaluate the effect of subjects age on strand-break repair, Singh et al. [22] employed the alkaline version to measure the DNA damage and repair in lymphocytes isolated from the peripheral blood of healthy subjects and exposed them in vitro to 2 Gy of X-radiation. For all subjects the mean level of DNA damage was restored to pre-irradiation control levels within 2 h of incubation at 37°C. However, due to the advantage of being able to analyze single cells, a distribution analysis of DNA damage among cells within each sample indicated the presence of a few highly damaged cells in the 2-h sample, the occurrence of which was significantly more common among aged individuals. Another important feature of this kind of strand break repair studies is the capacity for the recognition of individuals with an altered repair capability, Rojas et al. [108] found an important repair delay in lymphocytes from a healthy donor exposed to vanadium pentoxide.

Agents such as UV radiation that produce lesions which do not form strand breaks directly can be examined using the assay. Rather than detecting strand breaks produced by the irradiation, it is possible to detect strand breaks produced by the cell in its attempt to repair the lesion. With this approach Gedik et al. [46,250,251] using cells from individuals affected with Xeroderma pigmentosum, which are incapable to repair the UV damage, observed a non-induction of DNA strand breaks in this cells after irradiation, meanwhile in normal cells an increased DNA migration was observed after 1 h of irradiation. The assay has been suggested as a diagnostic tool for Xeroderma pigmentosum and other syndromes characterized by defects in excision repair [250].

The ability to detect excision repair sites using the alkaline version can be enhanced by the inclusion of repair inhibitors, DNA synthesis inhibitors or chain terminators such as *N*-hidroxyurea, aphidilcolin and cytosine arabinoside, respectively [250,252]. The enzymatic approach of Collins and co-workers can be used in the alkaline version to evaluate the repair kinetics of various classes of DNA lesions in treated cells. The UV-damage-specific T4 endonuclease can be used to study the removal of UV-induced pyrimidine dimers [250] while Endo III can be used to monitor the removal of oxidized pyrimidines [241]. Hydrogen peroxide induced damage levels which showed to be higher with endonuclease III treatment, demonstrating the persistence of oxidized base damage and therefore the differential expression of lesions following repair [253]. The oxidative and other DNA damage induced endogenously can be readily studied using experimental variations of the (SCGE) Comet assay because of its sensitivity. Apoptotic DNA fragmentation could be also studied [134]. Here again, the Comet assay provides a simple assay system with the advantage of allowing examination of the sequential steps of incision/excision and resynthesis/ligation.

3.4. Environmental biomonitoring

An optimal method for detecting genotoxicity damage in sentinel organisms should be capable of detecting many classes of damage in a variety of cell types from a range of organisms, provide data at the level of the individual cell and be sensitive, rapid and cost effective [14]. The (SCGE) Comet assay, again because of its simplicity, sensitivity, and need for only small number of cells has been suggested as an ideal technique for such studies.

To monitor for genotoxic pollutants at a hazardous site Nascimbeni et al. [254] conducted a pilot study

Table 3
Clinical applications

Agent	Cell type	Result	Refs.
4NQO	MRC5CV1 cells, 46BR cells, YBL6 cells, YBR/3 cells, GM 1492 cells, V79-171b cells, SCCVII cells, SiHa cells, WiDr cells	+	[176,180] [202,219]
4NQO	XP-12 Rosv cells	−	[176]
Acid black 48	Cells infected with HCMV	+	[220]
Acid blue-129	Cells infected with HCMV	+	[220]
Acid blue-40	Cells infected with HCMV	+	[220]
Actimomycin D	V79 cells, SiHa cells, WiDr cells	+	[202]
Alizarin	Cells infected with HCMV	+	[220]
AQ4N	Hypoxic tumor cells T50/80	+	[229]
ASA	HT 29 cells	+	[237]
Benzo(*a*)pyrene	Human colon cells, rat colon cells	−	[227]
Bladder carcinoma patients	Exfoliated cells from bladder (transitional cell carcinoma patients)	+	[191]
Bleomycin	V79 cells, TK-6 cells, WI-L2 cells, SB cells, Raji cells, GM0606 cells, MOLT4 cells, Jurkat cells, CCRF-HSB-2 cells, HL-60 cells, GM3440 cells, murine Friend erithroleukemia 707 cells, BUF cells, lymphocytes from breast cancer patients	+	[172,201,205] [206]
Blue-2	cells infected with HCMV	+	[220]
Cervical dysplastic	Cervical cells, peripheral blood leukocytes	+	[222]
Cisplatin	Cryopreserved human lymphocytes from patients with breast cancer	+	[163]
Clozapine	Human lymphocytes	+	[214]
Cyclophosphamide	Cryopreserved human lymphocytes from patients with breast cancer, human blood cells	+	[163,166,167]
Dexorubicin	V79-171b cells, SCCVII cells, SiHa cells, WiDr cells	+	[202,219]
DMBA	MRC5CV1 cells, XP-12 Rosv cells	+	[176]
DMH	Gastric tract of rat	+	[226]
DMH and lactobacillus	Gastric tract of rat	−	[226]
DNC	Human colon cells	+	[227]
Etoposide	WiDr colon carcinoma cells, V79 cells, SiHa cells, monolayers, spheroids and xenografted tumors in mice	+	[200–203]
H_2O_2	L5178Y subline murine lymphoma, LY-R, LY-S, human sperm from fertile and infertile men, Clone 707 Friend erythroleukemia cells	+	[191,215] [216,217]
Haloperidol	Human lymphocytes	+	[214]
Hexenal	Primary rat gastric mucosa, human lymphoblastoid Namalva cell line, primary rat colon mucosa, human primary esophagus mucosa, rat esophagus mucosa	+	[228]
Human mammary lipid	Human mammary epithelial cells	+	[236]
IL-1α	Human pancreatic cells	+	[238]
Interferon γ	Human pancreatic cells	+	[238]
Intestinal crypts	Intestinal crypts	+	[223]
Ischaemia-reperfusion-injury	Human white blood cells	+	[231]
LCA	Human colon cells, rat colon cells	+	[227]
MMS	V-E5 cells, XR-V15B cells	+	[235]

(Continued overleaf)

Table 3. Continued

Agent	Cell type	Result	Refs.
MNNG	Colon cells of rat, human colon cells, V79-171b cells, SCCVII cells, SiHa cells, WiDr cells, cervical cells, peripheral blood leukocytes	+	[202,219,222] [227,228]
MNNG and lactobacillus	Colon cells of rat	−	[226]
Morphine	HUT-78 cells	−	[218]
Morphine and EMS	HUT-78 cells	+	[218]
N-Methylsalsolinol	SH-SY5Y cells	+	[233,234]
NaCl prior radiation	HT 144 cells, HT 29 cells, DU145 cells, U87 cells	+	[198]
NCS	V-E5 cells, XR-V15B cells	+	[235]
Nicotinamide	C3H/Hen mice cells, SCCVII cells	+	[230]
Nicotinamide and radiation	Tumor hypoxic cells	+	[160]
Nitric oxide	Human pancreatic cells HIT, rat pancreatic cells	+	[224,225]
Oral squamous cells carcinoma grades I and III	Oral squamous cells from carcinoma	+	[165]
Phenotiazine	Human lymphocytes	+	[214]
PhIP	Human colon cells	−	[227]
PhIP	Rat colon cells	+	[227]
Precancerous and cancerous cervix lesions	Human cervical ephitelial cells, peripheral blood leukocytes	+	[221]
R-Salsolinol	SH-SY5Y cells	−	[233]
Radiation	Human estimulated and non-estimulated lymphocytes from Xeroderma pigmentosum patients, V79 cells, SCCVII tumor cells, C3H cells, TK-6 cells, WI-L2 cells, SB cells, Raji cells, GM0606 cells, MOLT4 cells, Jurkat cells, CCRF-HSB-2 cells, HL-60 cells, GM3440 cells, biopsies of breast cancer patients, Lewis lung cells, SiHa cells, KHT cells, RIF-1 tumors growing in mice, human T-lymphocytes, human fibroblasts, MRC5CV1 cells, HT-144 cells, DU-145 cells, U-87 cells, HIT-29 cells, hipoxic cells in solid tumors, 46BR cells, YBL6 cells, YBR/3 cells, L5178(R) cells, GM1492 cells, MeWo cells, PECA 4451 cells, PECA 4197 cells, melanocytic nevus cells, dysplastic nevus cells, human sperm from infertile male, C3H mammary tumors implanted into the feet of female CDF1 mice, Ataxia-telangiectasia cells, human lymphocytes from patients with thyroid tumors and healthy, HT1376 cells, UMUC-3 cells, RT112 cells	+	[156,159,160] [168–170] [171–173] [174–176] [177–179] [180–182] [183–185] [186–188] [189–191] [192–194] [195–197]
Radiation	XP-12 Rosv cells, XPD cells, TTD cells	−	[176,187]
RSU1069	Aerobic tumoral cells, SiHa cells, WiDr cells	+	[202,210]
S-Salsolinol	SH-SY5Y cells	−	[233]
SIN-1	HIT-T15 cells	+	[199]
Sodium iodide by oral administration	Peripheral blood cells	+	[239]
Sperm from fertile and infertile men	Human sperm from fertile and infertile men	−	[191]

Table 3. Continued

Agent	Cell type	Result	Refs.
Staurosporine	Human lymphocytes	+	[232]
Staurosporine and X-rays	Human lymphocytes	+	[232]
TMB-8	Human lymphocytes	+	[232]
TMB-8 and X-rays	Human lymphocytes	+	[232]
TNFα	Human pancreatic cells	+	[238]
Topoisomerase II (etoposide inhibitor)	V79-17b cells, Vpr cells	+	[204]
TPA	A 1698 bladder carcinoma cells, spleen, marrow tumoral cells, spheroids and murine tumors SCCVII, V79 cells, WiDr cells, SiHa cells, RIF-1 cells, EMT6 cellsA549 cells, HT29 cells, SiHa cells, WiDr cells, tumor-bearing mice	+	[202,207,209] [210–212] [213]
TPA	Cells from patients with Ataxia-telangiectasia	−	[208]
Violet R	cells infected with HCMV	+	[220]
White light and riboflavin	HL-60 cells	+	[177]
X/XO	A 1698 bladder carcinoma cells	+	[207]
X/XO	Cells from patients with Ataxia-telangiectasia	−	[208]

Table 4
DNA repair studies

Agent	Cell type	Result	Refs.
AraC	VH10 cells, HepG2 cells, V79 cells	+	[249]
Bleomycin	Bone marrow, testicular cells from Sprague–Dawley rat	+	[250]
Cyclophosphamide	Bone marrow, testicular cells from Sprague–Dawley rat	+	[250]
EGME	Bone marrow, testicular cells from Sprague–Dawley rat	+	[250]
EMS	Bone marrow, testicular cells from Sprague–Dawley rat	+	[250]
ENNG	CHO cells	+	[247]
ENU	CHO cells	+	[247]
GSNO	HIT-T15	+	[9]
H_2O_2	Human lymphocytes, HeLa cells, GM1899A cells,	+	[12,241–243]
HU	VH10 cells, HepG2 cells, V79 cells	+	[249]
MMS	HeLa cells, human lymphocytes	+	[12]
MNNG	VH10 cells, HepG2 cells, V79 cells	+	[249]
Ni subsulfide	MRC-5 cells	+	[248]
Oxidative damage	Human lymphocytes, HeLa cells	+	[249]
Radiation	CHO cells, V79 cells, TK6 repair deficient cells, HeLa cells, XRS-5-11 cells, V3 cells K-1 cells, AA8 cells, human blood granulocytes, human lymphocytes	+	[12,242,244–247]
Radiation and aphidilcoline	HeLa cells, human lymphocytes	+	[12,242]
Radiation and interferon α	Lymphocytes of whole blood, spleen and thymus from mice	+	[248]
SIN-1	HIT-T15	+	[9]

to assess the extent of DNA damage in different tissues of the golden mouse *Ochrotomys nuttalli*, live-trapped at a Superfund site in North Carolina, USA. The level of DNA damage, as measured by mean migration length, was increased in all tissues of animals from the hazardous waste site, but this increase was significant only in brain cells. Fairbain et al. [255] exposed Raji cells prepared using environmental water samples collected from various industrial sources in the Utah valley.

The alkaline version has also been used successfully to examine the extent of DNA damage in coelomocytes collected from earthworms maintained in different soil samples as an indicator of soil pollution [256,257]. Ralph and Petras have demonstrated the utility of the method for detecting increased levels of DNA damage in erythrocytes sampled from bullheads and carp collected at polluted sites around the great lakes [258] other fish species and invertebrate animals have also been used [14,259]. De Boeck and Kirsh-Volders [260] have used an annelid (*Nereis virens*) for the genotoxic evaluation of PAH exposure.

Another interesting application to the environmental biomonitoring is the approach used by Betti and Nigro [261] for the evaluation of genetic hazard of pollutants in cetaceans. Recently the adaptation of this technique to plant cells [41] opens new possibilities for studies in this area. The potential application of the (SCGE) Comet assay in environmental biomonitoring is almost unlimited, with any organism being suitable for investigation. For a more detailed reference of the data in this field, see Table 5.

Table 5
Environmental biomonitoring studies

Agent	Cell type	Result	Refs.
4NQO	Unicellular green alga	+	[272]
Atrex Nune-O	Tadpole erythrocytes	+	[267]
Basal damage in tadpoles naturally exposed	Whole blood from tadpole	+	[265]
Cd	Cells of root from *Vicia faba*	+	[266]
CH	Cells of root from *Vicia faba*	−	[266]
Cr	Cells of root from *Vicia faba*	+	[266]
Dual-960E	Tadpole erythrocytes	+	[267]
EMS	Cells of root from *Vicia faba*	+	[267]
Hazard waste site	Ochrotomys nutally	+	[254]
Hg	Dolphin lymphocytes	+	[270]
MMC	Cells of root from *Vicia faba*	+	[266]
MMS	Cells of root from *Vicia faba* erythrocytes from tadpole	+	[266]
N-Nitrosodimethylamine	Unicellular green alga	+	[272]
NT-spiked sediment	Cells of marine fish and invertebrates	+	[269]
Organic extracts of river sediments	Primary cultures of rainbow trout hepatocytes	+	[271]
PAH	Puncturing the coelomic cavity from *Polychaeta*	+	[272]
Pollutants in coke oven area	Coelomic leukocytes	+	[268]
Radiation	Bone marrow and stromal cells from canine, *Allium cepa* L. *roots*	+	[263,264,273]
Roundup	Tadpole erythrocytes	+	[267]
Sencor-500F	Tadpole erythrocytes	−	[267]
Water of differents lakes polluted with PAHs	Erythrocytes from fish	−	[262]
Water of differents lakes polluted with PCBs	Erythrocytes from fish	+	[262]

3.5. Human monitoring

An important application of the (SCGE) Comet assay is in human monitoring, assessing DNA damage in cells sampled from individuals exposed occupationally or environmentally.

The relevance of the SCGE in this area lies on its requirement of very small cell samples, its ability to evaluate DNA damage in non-proliferating cells and the fact that non-invasive procedures can be done to obtain sufficient numbers of cells from different tissues. Most human studies have evaluated DNA damage in nucleated blood cells [274]. The SCGE assay has been used in about 20 human monitoring studies (for information see Table 6), using mainly human lymphocytes samples from exposed individuals, data obtained has shown both positive and negative results. In a population study involving 200 healthy individuals Betti et al. [282] found that the extent of DNA migration measured using the alkaline version was significantly increased in blood lymphocytes of smokers, with a greater increase occurring in males than females. Frenzelli et al. [284] realized a follow-up of heavy smoking individuals that quit smoking and a year after quitting, and found a decrease in the value of DNA damage returning to similar values as non-smoking individuals. However other groups failed to detect differences between these groups. Sram et al. [291] reported lack of differences in DNA damage between mothers who smoke versus mothers that did not smoke in a Bohemian population.

The assay shows itself to be highly sensitive in detecting DNA damage under different treatments or conditions, Hartmann et al. [21] reported that physical activity above the aerobic–anaerobic threshold caused DNA damage in blood leukocytes, with the increase being detected 6 h after cessation, reaching a maximum at 24 h, and returning to control levels by 72 h. Recently the same group reported an increased DNA migration in leukocytes from all individuals which run a short distance triathlon, at different time points after exercise and revealed a biphasic pattern. Twenty-four h postexercise, elevated DNA migration was found, whereas lower values were detected 48 h after exercise. Seventy-two h postexercise, the maximum increase in DNA migration was found and baseline values were still elevated after 120 h. However, with the enzymatic approach for the detection of oxidized DNA bases they did not found any differences between the leukocytes before and after the triathlon, No differences were found in the micronucleus-frequency in lymphocytes before at 48 and 96 h after exercise [297]. This finding should not be interpreted as an indictment of exercise, but only that a very strenuous exercise can lead to secondary effects that are not originated from oxidized bases and do not result in chromosome damage.

Green et al. [87] demonstrated the value of a good breakfast (combined with vitamin C) by its ability to decrease consistently the in vitro sensitivity of lymphocytes to ionizing radiation, as measured by a decrease in DNA migration in cells irradiated with 2 Gy. The investigator concluded that variation in normal diet may not only disturb individual susceptibility to endogenous oxidative damage but may also affect individual responses to radiation. This results were later confirmed by others [66,298]. These kind of variables (physical activity, smoking habit and diet) could change the cellular response to irradiation or chemical exposition, and need to be considered in human monitoring studies applying the (SCGE) Comet assay. While cytogenetic biomonitoring studies are mainly done using lymphocytes, the SCGE assay can be applied to any cell population, giving an advantage for these kind of studies; however when studies involving whole blood are conducted it should be considered that, leukocytes are a heterogeneous mixture of cells, some populations having a life span which can vary from weeks to decades (i.e., B and T lymphocytes) while others (i.e., granulocytes) have a short half-life ranging from 7–24 h. These differences in lifespan among different cells are critical to the design and interpretation of biomonitoring studies and could explain the considerable intra-individual variation observed in some studies [298]. Several investigators have started collecting information about the different sensibility from granulocytes and lymphocytes exposed to γ-radiation as measured by migration length using the alkaline version [44]. Using the same version we were able to see differences in response (DNA migration length) to the same concentration of different metals in whole blood, isolated lymphocytes and G1-lymphocytes [108].

Table 6
Human studies

Population	Cell type	Result	Refs.
20 weeks with β-carotens supplementation	Human lymphocytes	−	[279]
20 weeks with vit C supplementation	Human lymphocytes	−	[279]
20 weeks with vit E supplementation	Human lymphocytes	−	[279]
Age from healthy subjects	Human lymphocytes	−	[282,283]
Air pollution	Nasal respiratory epithelium from children	+	[289]
Air pollution	Venous and cord blood	−	[291]
Air pollution (ozone)	Nasal epithelial cells, whole blood	+	[290]
Air pollution (ozone)	Buccal epithelial cells	−	[290]
Anti-CD38 in patients irradiated	Whole blood	−	[286]
Basal levels of oxidative damage	Human lymphocytes	+	[294]
Benzene	Human lymphocytes	+	[288]
Carotenoid	Human lymphocytes	−	[280]
Chronically irradiated volunteers	Blood cells	+	[293]
Dairy product-free diet	Human lymphocytes	−	[292]
Dairy product-rich diet	Human lymphocytes	+	[292]
Diets high risk	Colonic mucosa cells	+	[295]
Diets low risk	Colonic mucosa cells	+	[295]
Ex-smokers (1 year)	Human lymphocytes	−	[284]
Exercise	Peripheral white blood for humans	+	[277]
Exercise and multivitamin pills	Peripheral white blood for humans	+	[277]
Exercise and vit E supplementation	Peripheral white blood for humans	−	[277]
Infected and malnourished children	Human leukocytes, human lymphocytes	+	[276]
More than one hyperbaric oxygen therapy	Peripheral blood cells	−	[285]
Newborns and mothers passive smokers	Human lymphocytes	−	[275]
Newborns and mothers smokers	Human lymphocytes	+	[275]
Occupational exposure to oxidation hair dye	Human lymphocytes	−	[296]
One hyperbaric oxygen therapy	Peripheral blood cells	+	[285]
Physical activity in aerobic conditions	Peripheral blood cells	+	[21]
Physical activity in anaerobic conditions	Peripheral blood cells	−	[21]
Rubber industry workers	Peripheral blood lymphocytes	+	[287]
Smoke habit from healthy subjects	Human lymphocytes	+	[282,283]
Smokers	Buccal epithelial cell	+	[16]
Styrene workplace	Human T-lymphocytes from blood	+	[281]
Vit C supplementation	Nucleated cells of blood	−	[87]
Vit C supplementation in individuals with low or high cholesterol levels	Human blood lymphocytes	−	[278]
Vit C with breakfast	Nucleated cells of blood	−	[87]

Using a cell type sorter based on differences in membrane markers, Uzawa et al. [299] demonstrated that CD45RO$^+$ memory cells were more radiosensitive in vitro than CD45RO$^-$ naive T cells. Strauss et al. [300] introduced an immunological typing technique capable of identifying the subtypes of blood leukocytes directly in the gel matrix. This approach has been used by Tice and Strauss [4] to demonstrate comparable repair kinetics for single strand breaks induced in B, T, T-helper and T-supressor cells irradiated with 1.75 Gy of γ rays.

Taking advantage of the SCGE characteristics other cells have been used in monitoring studies, Calderon et al. [289] obtained cells from nasal epithelial biopsies from children and individuals that live in Mexico city, and observed an increase in the number of cells with comets in persons that live in the city. Furthermore we were able to adapt the SCGE to buccal epithelial cells showing that smokers have clearly more damage than non-smokers in these cells, the technique was able to detect differences in smokers who smoked more than 10 cigarettes daily [16]. However with this method we did not find differences in DNA damage among the buccal epithelial cells from individuals who live in the south and north part of Mexico City, exposed to air pollution (principally ozone), although we observed a statistically difference between these groups in nasal and whole blood cells [290].

An exciting new approach to human monitoring studies has been introduced by Collins et al. [12]. This group added different enzymes to evaluate the oxidized bases. With this approach they studded the basal level of oxidized base damage in human lymphocytes [301].

The use of this assay for human studies is at this moment controversial, because of the great variability observed in both controls and exposed groups mainly in the studies using leukocytes. This could be due to problems attributable to the technique, although it can also be explained by the dose or concentration and the exposure route. However with the enzymatic approach and a better delimitation of the cell type subpopulations it will be possible to assess better the DNA damage. On the other hand the confounding factors to be taken into consideration for the assay when used in this area are the same as those in other human monitoring studies. It is known, that controls and exposed individuals need to be in the same physiological state at the time of sampling.

The (SCGE) Comet assay in human studies, especially when they are combined with the ability to identify selected cell populations and to recognize different classes of DNA damage using specific enzymes will be of great utility for understanding chemicals that are able to impair human health.

4. Future directions and conclusions

The (SCGE) Comet assay has demonstrated its sensitivity as a technique for the evaluation of DNA damage among a variety of cell types, induced by a variety of physical and chemical agents. In comparison with other sensitive methods, the Comet assay is relatively robust and economical in its use of material.

To be precise, the (SCGE) Comet assay detects the release of DNA from a highly supercoiled DNA–protein complex. In this respect it is similar to other sensitive methods for detecting DNA strand breakage in mammalian cells, including DNA precipitation, alkali elution, alkali unwinding and nucleoid sedimentation. Although all these techniques except SCGE, use similar lysis procedures and any method sufficiently rigorous to remove all associated protein would be likely to introduce too many strand breaks in controls to achieve useful sensitivity in mammalian cells [9].

The advantages of the technique include: (1) data is collected at the level of the individual cells, (2) only a small number of cells is required, (3) almost any eukaryotic cell population can be used, (4) the assay is sensitive, simple and cost effective, (5) data can be obtained within a few hours of sampling, (6) the assay can evaluate DNA damage in non-proliferating cells and (7) it has the specific advantage that as a single-cell assay, it can detect non-uniform responses within a mixed population.

The biological significance of the test is not as yet firmly defined. The DNA damage detected by the (SCGE) Comet assay can arise through various mechanisms, including DNA double- and single-strand breaks, DNA interstrand cross-linking, alkali labile sites, and incompletely repaired excision sites

present at the time of lysis. The alkaline version can be modified in such a way that specific classes of damage in selected cell types can be easily investigated. A positive response in the assay means that the above events have been detected.

The potential applications of the (SCGE) Comet assay in such areas as genotoxicity, clinical, DNA repair, environmental biomonitoring and human monitoring is almost unlimited. However we are in agree with Tice [10] that due to its easy application it ensures that the assay will be misused and the resulting data misinterpreted. To minimize such occurrences, several important issues need to be addressed.

A new exciting approach has been introduced by McKelvey-Martin et al. [197] adapting the fluorescent in situ hybridization to the (SCGE) Comet assay methodology, opening a new opportunity to identify gene breakage or gene amplification and DNA damage in the same slide.

The future applications of the (SCGE) Comet assay could impact some other important areas, certainly, one of the limiting factors to its utility is the imagination of the investigator.

5. List of abbreviations

2AF	2-Amino fluorene
4NQO	4-Nitroquinolone-*N*-oxide
4Q4N	{1,4-bis-([2-(dimethylamino-*N*-oxide)-ethyl] amino)5,8-dihydroxyanthracene-9,10-dione}
5-FU	5-Fluorouracil
8-OH gua	8-Hydroxyguanine
8HQ	8-Hydroxyquinoline
8HQ	8-Hydroxyquinoline
AHH	Arylhydrocarbon hydroxylase
Ara C	Cytosine arabinoside
ASA	Aspirin
AZQ	2,5-Diaziridinyl-3,6-bis(carboethoxy-amino)-1,4-benzoquinone
BHC	Benzene-1,2,3,4,5,6-hexachloride
BPDE	Benzo[*a*]pyrene diol epoxide
BZQ	2,5-Diaziridinyl-3,6-bis(ethanolamino)-1,4-benzoquinone
Cd	Cadmium
Cr	Chromium
CH	Cyclohexamide
DAB	*p*-Dimethylaminoazobenzene
DAPI	4,6-Diamidino-2-phenylindole
DMBA	7,12-Dimetylbenzo[*a*]anthracene
DMH	1,2-Dimethyñhydracine
DMSO	Dimethyl sulfoxide
DNC	Dinitrosocaffeidine
EDTA	Ethylenediaminetetraacetic acid
Endo III	Endonuclease III
ENNG	Ethyl-*N*-nitroso guanidine
ENU	*N*-Nitoso-*N*-ethylurea
ETU	Ethylene thiourea
Fpg	Formamido pyrimidine glycosylase
GSNO	*S*-Nitrosoglutathione
Gy	Gray
H	Hour
H_2O_2	Hydrogen peroxide
Hg	Mercury
HU	Hydroxyurea
INO2	1-Methyl-2-nitroimidazole
IQ	2-Amino-3-methyl-3H-imidazo[4,5*f*]-quinoline
LCA	Lithocholic acid
MeIQ	Heterocyclic amine
MeIQx	2-Amino-3,8-dimethylimidazol[4,5*f*]-quinoline
MMC	Mitomycin C
MMS	Methylmethane sulphonate
MNNG	*N*-Methyl-*N*-nitro-*N*-nitrosoguanidine
NaCl	Sodium chloride
NCS	Neocarzinostatin
NMU	*N*-Nitroso-*N*-methyl urea
NSAID	Non-steroidal anti-inflamatory drug
OPP	*ortho*-Phenyl phenol
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PhIP	2-Amino-1-methyl-6-phenylimidazo-[4,5*b*]pyridine
PK	Proteinase K
SCGE	Single cell gel electrophoresis
SIN-1	3-Morpholinosydomine
SOD	Superoxide dismutase
TBZ	2-(4-Thiazolyl)benzimidazole
TGFβ	Factor growing tumor beta
TNFα	Factor necrosis tumor alpha
TPA	3-Amino-1,2,4-benzotriazine-1,4-dioxide
Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido-[4,3-*b*]indole

Trp-P-2	3-Amino-1-methyl-5H-pyrido-[4,3-*b*]ondole
Uvra	Ultraviolet radiation A
Uvrb	Ultraviolet radiation B
Uvrc	Ultraviolet radiation C
Vit	Vitamin
WC–Co	Cobalt–tungsten carbide
X/XO	Xanthine/xanthine oxidase
YOYO-1	Benzoxazolium-4-quinolinum oxazole yellow homodimer

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