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Standardisation of the *in vitro* comet assay: influence of lysis time and lysis solution composition on the detection of DNA damage induced by X-rays

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Abstract

The alkaline comet assay, *in vivo* and *in vitro*, is currently used in several areas of research and in regulatory genotoxicity testing. Several efforts have been made in order to decrease the inter-experimental and inter-laboratory variability and increase the reliability of the assay. In this regard, lysis conditions are considered as one of the critical variables and need to be further studied. Here, we tested different times of lysis (from no lysis to 1 week) and two different lysis solutions in human lymphoblast (TK6) cells unexposed or exposed to X-rays. Similar % tail DNA values were obtained independently of the time of lysis employed for every X-ray dose tested and both lysis solutions. These results, taken together with our previous ones with methyl methanesulfonate and H_2O_2, which showed clear lysis-time dependence, support that the influence of the lysis time in the comet assay results depends on the type of lesion being detected; some DNA lesions may spontaneously give rise to apurinic or apyrimidinic (AP) sites during the lysis period, which can be converted into strand breaks detectable with the comet assay. Testing different times of lysis would be useful to increase the sensitivity of the comet assay and to ensure the detection of DNA lesions of an unknown compound, thereby providing some insight into the chemical nature of the lesions induced. However, the same lysis conditions (i.e. lysis time and lysis solution) should be used when comparing results between different experiments or laboratories.

Introduction

Although the initial description of the comet assay appeared in 1984 (1), it was not until 1988 that the most widely used protocol was described (2), by increasing the alkalinity of the electrophoresis buffer to pH >13. The technique is in principle applicable to any tissue from which single cells/nuclei can be obtained. It is easily implemented in laboratories, quick and economical. Due to its versatility, it has been used in many research areas during all these years, including genotoxicity testing (both *in vitro* and *in vivo*), human biomonitoring, ecogenotoxicology and in basic research (3).

Under alkaline conditions (pH > 13), the comet assay is able to detect single and double strand breaks, as well as alkali-labile sites (ALS). Moreover, its ability to detect a broader spectrum of primary DNA alterations is another argument in its favour; by combining it with specific endonucleases, other lesions (e.g., oxidised and alkylated bases, misincorporated uracil and pyrimidine dimers) can also be detected (4–6). In addition, quantifying the extent of DNA damage is possible since the comet assay has been calibrated from the earliest papers against the known strand-breaking effects of X-(2) or γ-irradiation (1); specifically, 1 Gy of X- or γ-irradiation introduces 0.31 breaks per 10^9 Daltons of DNA (7).

The *in vivo* comet assay is part of the strategy suggested by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (8) and it is also contemplated by the European Food Safety Authority (EFSA) for genotoxicity testing of compounds in food and animal feedstuffs (9). The corresponding OECD guideline (OECD TG 489) was first approved in 2014 with its last version adopted on 29th July 2016 (10). The *in vitro* version of the assay is widely used for genotoxicity screening of novel cosmetics, nanomaterials and pharmaceuticals, and is recommended as an appropriate test for use under the Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) programme of the European Commission. The European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) implemented an initiative to evaluate the validity of the *in vitro* comet assay (11). However, this validation study is currently stopped (12). On the other hand, a validation study of the comet assay in a reconstructed human 3D skin model, coordinated by Cosmetics Europe, is still ongoing (12).

Although the comet assay has been extensively used for almost 30 years now, its role in regulatory genotoxicity assessment has been limited. This might be due not only to the fact that the assay detects pre-mutagenic DNA lesions rather than fixed mutations, but also to the high variation and thereby limited reliability of the results reported. Indeed, the high intra-assay variability and low inter-assay reproducibility (13) made it necessary to identify and study the critical points of both the *in vivo* and *in vitro* versions of the assay.

Some important factors that influence the outcome of the *in vitro* as well as the *in vivo* comet assay have already been identified: final agarose concentration in gels (14,15), duration of the alkaline unwinding treatment (14–16), electrophoresis conditions (14,15,17,18), enzyme-incubation time (15) and DNA staining (19), are all critical points affecting the results obtained. Conditions of the lysis step may also affect the comet assay results. In this step, membranes and soluble cytoplasmic and nuclear components of the cells are removed by treating with a high-salt concentration and detergent (20), leaving the DNA attached at intervals to the nuclear matrix (the so-called 'nucleoids'). However, few studies regarding the effect of lysis conditions are available. Most comet assay protocols, including the *in vivo* OECD guideline, agree that at least 1 h of lysis should be used (7,10,21). Although the OECD guideline also suggests longer (i.e. overnight) lysis periods, it recommends keeping the lysis conditions as constant as possible for all the samples within an experiment (10). Nevertheless, since 1990, very few researchers perform cell lysis and DNA unwinding at the same time using an alkaline solution (pH > 13) (19,22–24).

We recently evaluated the effect of modifying the time of lysis in untreated and methyl methanesulfonate (MMS)- or H_2O_2-treated HeLa cells on the standard alkaline comet assay results, as well as in untreated and Ro 19-8022 plus light-treated HeLa cells on the alkaline comet assay combined with formamidopyrimidine-DNA-glycosylase (Fpg) results (25). As demonstrated, different times of lysis (i.e. from no lysis to 1 week of lysis) strongly affected the results of both the standard alkaline comet assay and when combined with Fpg. In this regard, a constant time of lysis was recommended to reduce inter-experimental variability and to facilitate comparison of results between experiments. However, varying the time of lysis could also be used to study the nature and the frequency of DNA lesions induced by genotoxic chemicals inducing different types of DNA lesions.

In our previous paper (25), we suggested that different cell lines and different types of lesions may require different conditions in order to achieve the desired sensitivity. It is well known that some cell types such as human or animal keratinocytes (26) and human buccal cells (27) need extensive lysis including digestion with proteinase K to remove residual proteins. Moreover, dithiothreitol is also used in order to decondense the tightly packed chromatin of sperm (28).

Comparison of results from different laboratories would benefit from standardising the critical steps of the comet protocol, including the lysis step. Moreover, different lysis solution compositions are used. According to our knowledge, there is no study addressing the influence of the lysis solution composition on the comet assay results. Thus, the aim of this work was to test the effect of modifying not only the time of lysis, but also the composition of the lysis solution in the standard alkaline comet assay in human lymphoblast (TK6) cells, unexposed or exposed to different doses of X-rays.

Materials and methods

Cells

TK6 cells (human-derived lymphoblastoid cell line) originally from the American Type Culture Collection (ATCC) were used. Cells were thawed using standard procedures and grown in RPMI-1640 medium with L-glutamine supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C, according to ATCC's recommendations.

X-ray treatment

Cells were counted, centrifuged at 390×*g* at room temperature for 5 min and resuspended in Dulbecco's phosphate buffered saline (PBS) at 37°C to obtain a cellular concentration of approximately 1 × 10^6 cells/ml. Then, aliquots of 100 µL were prepared for X-ray exposure and put on ice. Cell aliquots were irradiated on ice/water with 4 or 8 Gy of X-rays (225 KV filtered through 0.5 mm Cu, 13 mA, 3.82 Gy/min dose rate), followed by comet analysis (see next section). A negative control (unexposed cells) cell aliquot was included in every experiment. Appropriate X-ray doses, for evaluation of lysis time and lysis solution composition were selected from a dose–response experiment ranging from 0 to 12 Gy, employing 1 hour of lysis with lysis solution A (see next section).

Comet assay

After irradiation, cells were also kept on ice until further processing. Ten microlitres of cell suspensions were mixed with 90 µl of 0.75% low melt point agarose in PBS with EDTA (10 mM) at 37°C, and 4 µl cell/agarose mixtures were immediately placed on GelBond® Films using an electronic multichannel pipette (two films were made for each lysis time tested), as described in (29). Lysis at 4°C was omitted, or performed by immersing the films in lysis solution A (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-base, pH 10.0, 1% Triton X-100 prior to use) or lysis solution B (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris-base, 34 mM N-Lauroylsarcosine sodium salt, pH 10.0, 1% Triton X-100 and 10% DMSO prior to use) for 1, 24 h or 1 week. Films were then rinsed in cold distilled water, and alkaline unwinding was performed by immersing them in an alkaline solution (0.3 M NaOH, 1 mM Na_2-EDTA, pH > 13) at 4°C for 40 min. Twenty-five minutes electrophoresis was performed in fresh alkaline solution at 0.85 V/cm and 10°C, with circulation of the solution at the rate of 10% of the volume per minute. Afterwards, films were rinsed in distilled water, neutralised in PBS for 10 min, rinsed again in distilled water and washed twice in

absolute ethanol (5 min in previously used ethanol + 90 min in fresh ethanol), before allowing them to dry overnight.

For scoring, DNA in each GelBond® Film was stained by immersing in SYBR® Gold (Invitrogen™ SYBR™ Gold Nucleic Acid Gel Stain, 10 000× concentrate in DMSO) diluted 1:10 000 in TE-buffer (1 mM Na_2-EDTA, 10 mM Tris–HCl, pH 8.0) (Stock solutions of 1:10 in DMSO were prepared and stored at −20°C). Films were covered when placed on a tilting shaker and allowed to stain for 20 min. After staining, films were rinsed in distilled water, and comets were visualised under a fluorescence microscope (Leica DMLB). DNA damage was quantified in 100 randomly selected comets per sample from two duplicate gels (50 comets in each gel) by measuring the % tail DNA using the image analysis software Comet Assay IV (Perceptive Instruments Ltd). For each treatment, the median value of the % tail DNA was calculated.

In addition, pH of lysis solutions A and B was monitored in an independent study after 1, 24 h and 1 week at 4°C.

Statistics

Three independent experiments were carried out in each case and the means of the median values of % tail DNA and standard deviations were calculated. Linear regression was calculated for the dose–response, and non-parametric one-way analysis of variance (i.e. Kruskal–Wallis test) was performed to compare the results obtained with different times of lysis and the two lysis solutions used.

Results

The results of the X-ray dose–response curve obtained with the comet assay in TK6 cells after 1 h of lysis in lysis solution A are shown in Figure 1. A dose–response with high linear correlation between X-ray dose and DNA damage (R^2 = 0.9892) was obtained with doses ranging from 0 to 12 Gy (with 2 Gy intervals). To test the effect of the time of lysis and the composition of the lysis solution on the comet assay results, we exposed TK6 cells to 0, 4 and 8 Gy, thereby producing a wide range of DNA damage.

When comparing the effect of different lysis periods (no lysis, 1, 24 h and 1 week), similar % tail DNA values were obtained for each X-ray dose independently of the time of lysis or the composition of the lysis solution used (Figure 2). Similar results were obtained for both unexposed and X-ray-exposed TK6 cells either omitting the lysis step, or lysing them for 1, 24 h or 1 week.

The pH of both lysis solutions slightly decreased over time.

Discussion

The alkaline version of the comet assay (the most used procedure nowadays) has been extensively used since it was first described in 1988 (2), with increasing efforts to standardise the protocol over the past few years, thereby trying to avoid inappropriate comparison of results. As mentioned in the introduction, several critical parameters, such as the final agarose concentration in gels, duration of the alkaline unwinding treatment, electric potential and duration of the electrophoresis, enzyme incubation (in case of combining the assay with enzymes) and staining, have been already addressed in various studies (14–19).

The time of lysis also seems to have a key role in the results obtained both with the standard alkaline comet assay and the comet assay in combination with Fpg (25). In our previous work, we examined the effect of modifying the time of lysis in untreated and MMS- or H_2O_2-treated HeLa cells in the standard alkaline comet assay, as well as in untreated and Ro 19-8022 plus light-treated HeLa cells in the alkaline comet assay combined with Fpg (25). The time of lysis did not affect the low levels of damage in untreated cells, but it had a marked effect in H_2O_2- and MMS-treated cells (Table 1, using lysis solution A). Interestingly, similar DNA lesion levels were obtained either skipping the lysis step or after 1 h of lysis, but a lower assay sensitivity was observed in comparison with longer times of lysis (from 24 h to 1 week of lysis). Regarding Ro 19-8022 plus light-treated cells, the comet assay was performed in combination with Fpg. In this case, 5 min of lysis was enough to detect oxidised bases with Fpg, and no differences were observed when comparing 5 min and 1 h of lysis. On the other hand, after 24 h of lysis, the frequency of Fpg-sensitive sites increased not only in Ro-treated cells but also in the negative control, though the increase was higher in treated cells (25). Finally, similar DNA lesion levels were obtained after either 24 h or 1 week of lysis (Table 1).

In the present work, we chose to introduce a defined and well-characterised set of DNA lesions by exposing cells to X-rays. We therefore expanded the array of DNA lesions investigated with respect to lysis conditions in the alkaline comet assay, to further understand the importance of the type of lesion. Exposing TK6 cells to X-rays resulted in a quite constant % tail DNA, unaffected by the time of lysis employed, for every X-ray dose tested (Figure 2; Table 1). However, solution B gave more constant results in this set of experiments, so it could be interesting to further study this effect.

When using the standard comet assay (i.e. without enzymes), the results obtained without lysis or with 1 h of lysis are quite similar (Figure 2; Table 1), which is in agreement with our previous findings (Table 1) (25), suggesting that the alkaline treatment is sufficient to lyse the cells. This is in agreement with the comet assay protocol

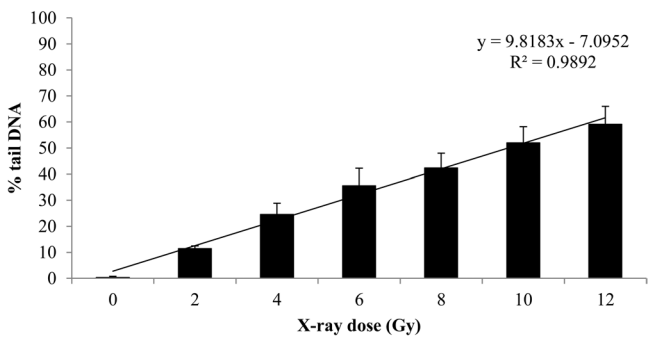

Fig. 1. X-ray dose–response obtained with the comet assay in TK6 cells after 1 hour of lysis with lysis solution A. Mean ± SD obtained in three independent experiments are represented.

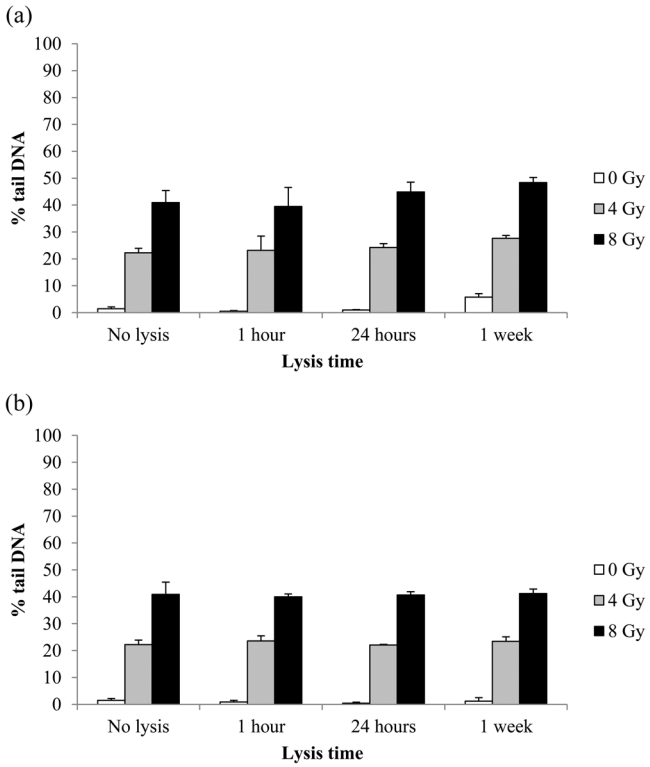

Fig. 2. Effect of different times of lysis on the DNA damage detected by the comet assay in TK6 cells unexposed or exposed to different X-ray doses with lysis solution A (a) or lysis solution B (b). Mean ± SD obtained in three independent experiments are represented.

Table 1. % tail DNA values obtained with increasing times of lysis when the comet assay was applied to TK6 cells unexposed or exposed to X-rays (*data obtained for lysis solutions A and B are the same since no lysis was performed) and to HeLa cells untreated or treated with MMS, H_2O_2 or Ro 19-8022 + light (**Data taken from (23); #the comet assay was performed in combination with Fpg)

DNA-damaging agent	Cell line	Dose	Lysis solution	Time of lysis			
				No lysis	1 h	24 h	1 week
X-ray	TK6	0 Gy	A	1.5 ± 0.7	0.5 ± 0.2	1.0 ± 0.2	5.8 ± 1.3
		4 Gy		22.2 ± 1.7	23.1 ± 5.3	24.3 ± 1.4	27.6 ± 1.1
		8 Gy		40.9 ± 4.5	39.5 ± 7.1	44.9 ± 3.6	48.4 ± 1.9
X-ray	TK6	0 Gy	B	1.5 ± 0.7	0.8 ± 0.6	0.5 ± 0.3	1.2 ± 1.3
		4 Gy		22.2 ± 1.7	23.5 ± 2.0	22.1 ± 0.2	23.4 ± 1.7
		8 Gy		40.9 ± 4.5	40.0 ± 1.1	40.7 ± 1.2	41.2 ± 1.7
MMS (**)	HeLa	0 µM	A	3.3 ± 2.2	3.1 ± 3.0	1.4 ± 1.1	2.5 ± 2.4
		90 µM		14.4 ± 2.6	12.2 ± 4.7	22.2 ± 2.7	55.0 ± 14.4
		180 µM		38.5 ± 14.6	23.7 ± 2.2	44.7 ± 6.0	81.5 ± 8.5
H_2O_2 (**)	HeLa	0 µM	A	5.7 ± 2.4	2.4 ± 1.8	1.9 ± 1.5	3.4 ± 0.1
		10 µM		13.3 ± 7.1	8.8 ± 0.4	16.6 ± 6.7	33.4 ± 0.2
		40 µM		42.4 ± 3.1	40.1 ± 1.6	63.5 ± 4.9	80.7 ± 6.8
Ro 19-8022 + light (**)	HeLa	0 µM	A	0.9 ± 1.1	5.2 ± 2.4	12.9 ± 0.7	13.0 ± 2.2
		1 µM		0.0 ± 0.0	34.4 ± 3.7	61.4 ± 8.2	60.5 ± 6.9

Mean ± SD obtained in three independent experiments are represented.

used by Olive and colleagues (19,22–24). Therefore, depending on the desired assay sensitivity and the genotoxic treatment employed, the lysis step could be even skipped. Vivek Kumar *et al.* (30), evaluated whether the alkaline treatment of cells could replace the lysis step in control and γ-irradiated lymphocytes. They found a good correlation between both protocols (i.e. with and without the lysis step), but also a slightly decreased sensitivity when the lysis step was removed, maybe due to a rather short alkaline unwinding period (20 min instead of the conventional 40 min used in our studies). Moreover, they included 0.02 M Trizma® base to the alkaline solution to avoid an increase in the background levels of DNA damage in control cells when the lysis step was not performed. In some cases, we have also observed a slight increase when the lysis step is omitted.

The time of lysis is not critical when detecting X-ray-induced DNA damage. This demonstrates that the time of lysis has a different effect on the outcome of the assay depending on the type of lesion being

detected. MMS is a monofunctional alkylating agent that directly attacks nitrogen and oxygen atoms of DNA bases and the oxygen moieties of the phosphate backbone (i.e. nucleophilic sites), mainly producing 7-methylguanine (31), and also generating some reactive oxygen species (ROS) (32). Ro 19-8022 is a photosensitiser which, in combination with light exposure, generates oxidative DNA modifications [mostly 8-hydroxyguanine (8-oxoG) residues] via generation of ROS, in particular singlet oxygen ($^1O_2$) (33). H_2O_2 is a potent oxidant compound generating ROS (i.e. hydroxyl radicals by the Fenton reaction in the presence of transition metals (34)), which can produce base modifications, degradation products of deoxyribose and chain breaks (35). X-rays induce DNA lesions directly, as DNA single strand breaks, AP sites and a few double strand breaks, as well as indirectly, by the production of short lived ROS (predominantly superoxide anions, hydrogen peroxide and hydroxyl radicals) that lead to an array of base modifications (e.g. oxidised pyrimidines) (36). N-Alkylated and oxidised bases are mainly recognised and hydrolysed by specific DNA N-glycosylases in the first step of the base excision repair (BER) pathway, thus leading to apurinic/apyrimidinic (AP) sites, which can be converted into strand breaks, either through the alkaline treatment as they are alkali-labile (37), or at neutral conditions via the AP-lyase activity of bifunctional DNA N-glycosylases. In addition, N-methylations can also lead to AP site formation by spontaneous glycosylic bond labilisation (37,38). The increase in the % tail DNA observed for some genotoxicants with longer times of lysis might correspond to spontaneously formed AP sites, similar to those originating from N-methylations, arising during the lysis step (pH 10) from both alkylated and oxidised bases. This hypothesis can explain the increased % tail DNA obtained with longer times of lysis in the case of MMS- or H_2O_2-treated cells. Nevertheless, we have not detected an increase in the % tail DNA of X-ray treated cells when increasing the time of lysis, likely to be due to the fact that 8-oxo-gua, the most frequent oxidized lesion induced by X-rays, is not converted into ALS under the lysis conditions tested.

Although more investigations will be needed to understand the underlying mechanism of the effect of the lysis time on the comet assay results, the *In Vivo* Mammalian Alkaline Comet Assay OECD Guideline already recognises conditions of the lysis step as a critical variable which may interfere with the level of strand breaks detected resulting from specific types of DNA modifications (10). This implies that the composition of the lysis solution may also affect the outcome of the assay. In this regard, similar results were obtained when performing the experiments using lysis solution A (Figure 2A) or lysis solution B (Figure 2B), although the results obtained with lysis solution B were more constant. The difference between the solutions is the presence of 34 mM N-Lauroylsarcosine sodium salt and 10% DMSO in lysis solution B. The slightly and not significant decreased % tail DNA obtained with lysis solution B for both non-irradiated and X-ray-irradiated cells after 24 h and 1 week of lysis may be ascribed to a protective effect of DMSO in lysis solution B.

As described in the introduction, experiments with some cell types require specific lysis conditions, both with respect to the lysis solution composition and lysis duration, in order to correctly measure their DNA damage levels. In the present work, we have used non- and X-irradiated TK6 cells while in our previous work we used untreated and MMS-, H_2O_2- and Ro 19-8022 + light-treated HeLa cells (Table 1). Though, it is most unlikely that TK6 and HeLa cells would differ in their lysis requirements, potential cell-type differences should be considered.

To sum up, no significant differences in DNA damage levels induced by X-rays in TK6 cells were detected with the alkaline comet assay regardless of the time of lysis and the lysis solution employed. These results, together with our previous studies, support the hypothesis that the influence of the time of lysis depends on the type(s) of DNA lesions induced. When unknown DNA lesions are to be measured (i.e. when testing a new compound), it is recommended to test different times of lysis in order to optimise the sensitivity of the comet assay and to ensure the detection of DNA lesions, thereby also providing some insight into the chemical nature of the lesions induced. Taking into account our results (Table 1), and in order to maximise the detection of the lesions, we would suggest no lysis or 1 h of lysis for X-ray treated cells, 1 week of lysis for MMS and H_2O_2 treatments and 24 h for Ro 19-8022 plus light-treated cells. On the other hand, the same time of lysis, as well as the same lysis solution, should be used in order to compare results between different experiments or laboratories.

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References

1. Ostling, O. and Johanson, K. J. (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.*, 123, 291–298.
2. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, 175, 184–191.
3. Azqueta, A. and Collins, A. R. (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch. Toxicol.*, 87, 949–968.
4. Azqueta, A., Arbillaga, L., López de Cerain, A. and Collins, A. (2013) Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG. *Mutagenesis*, 28, 271–277.
5. Collins, A. R. (2017) The use of bacterial repair endonucleases in the comet assay. *Methods Mol. Biol.*, 1641, 173–184.
6. Olsen, A. K., Duale, N., Bjørås, M., Larsen, C. T., Wiger, R., Holme, J. A., Seeberg, E. C. and Brunborg, G. (2003) Limited repair of 8-hydroxy-7,8-dihydroguanine residues in human testicular cells. *Nucleic Acids Res.*, 31, 1351–1363.
7. Collins, A. R., Oscoz, A. A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C. C. and Stetina, R. (2008) The comet assay: topical issues. *Mutagenesis*, 23, 143–151.
8. ICH S2(R1). (2012) *The tripartite harmonised ICH Guideline S2(R1): guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use*. http://www.ich.org/products/guidelines/safety/ article/safety-guidelines.html.
9. EFSA Scientific Committee. (2011) Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA J.*, 9, 2379. doi:10.2903/j.efsa.2011.2379.

10. OECD. (2016) OECD Guidelines for the testing of chemicals. *In vivo mammalian alkaline comet assay*. OECD Test Guideline 489. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.
11. Burlinson, B., Tice, R. R., Speit, G., *et al*.; In Vivo Comet Assay Workgroup, part of the Fourth International Workgroup on Genotoxicity Testing. (2007) Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup. *Mutat. Res*., 627, 31–35.
12. EURL-ECVAM. (2014) *EURL ECVAM status report on the development, validation and regulatory acceptance of alternative methods and approaches*. http://publications.jrc.ec.europa.eu/repository/handle/JRC90989. doi:10.2788/86047.
13. Møller, P., Möller, L., Godschalk, R. W. and Jones, G. D. (2010) Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group. *Mutagenesis*, 25, 109–111.
14. Azqueta, A., Gutzkow, K. B., Brunborg, G. and Collins, A. R. (2011) Towards a more reliable comet assay: optimising agarose concentration, unwinding time and electrophoresis conditions. *Mutat. Res*., 724, 41–45.
15. Ersson, C. and Möller, L. (2011) The effects on DNA migration of altering parameters in the comet assay protocol such as agarose density, electrophoresis conditions and durations of the enzyme or the alkaline treatments. *Mutagenesis*, 26, 689–695.
16. Yendle, J. E., Tinwell, H., Elliott, B. M. and Ashby, J. (1997) The genetic toxicity of time: importance of DNA-unwinding time to the outcome of single-cell gel electrophoresis assays. *Mutat. Res*., 375, 125–136.
17. Vijayalaxmi, Tice, R. R. and Strauss, G. H. S. (1992) Assessment of radiation-induced DNA damage in human blood lymphocytes using the single-cell gel electrophoresis technique. *Mutat. Res. Mutagen. Relat. Subj*., 271, 243–252.
18. Speit, G., Trenz, K., Schütz, P., Rothfuss, A. and Merk, O. (1999) The influence of temperature during alkaline treatment and electrophoresis on results obtained with the comet assay. *Toxicol. Lett*., 110, 73–78.
19. Olive, P. L., Banáth, J. P. and Durand, R. E. (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat. Res*., 122, 86–94.
20. Azqueta, A. and Collins, A.R. (2011) the comet assay: a sensitive and quantitative method for analysis of DNA damage. *Encycl. Anal. Chem*. doi:10.1002/9780470027318.a9192.
21. Tice, R. R., Agurell, E., Anderson, D., *et al*. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen*., 35, 206–221.
22. Olive, P. L., Wlodek, D., Durand, R. E. and Banáth, J. P. (1992) Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp. Cell Res*., 198, 259–267.
23. Banáth, J. P., Kim, A. and Olive, P. L. (2001) Overnight lysis improves the efficiency of detection of DNA damage in the alkaline comet assay. *Radiat. Res*., 155, 564–571.
24. Olive, P. L. and Banáth, J. P. (2006) The comet assay: a method to measure DNA damage in individual cells. *Nat. Protoc*., 1, 23–29.
25. Enciso, J. M., Sánchez, O., López de Cerain, A. and Azqueta, A. (2015) Does the duration of lysis affect the sensitivity of the in vitro alkaline comet assay? *Mutagenesis*, 30, 21–28.
26. Geffard, A., Doucet, O., Decome, L. and Me, M. D. (2005) Evaluation of photolyase (Photosome Ò) repair activity in human keratinocytes after a single dose of ultraviolet B irradiation using the comet assay. 79,101–108.
27. Rojas, E., Valverde, M., Sordo, M. and Ostrosky-Wegman, P. (1996) DNA damage in exfoliated buccal cells of smokers assessed by the single cell gel electrophoresis assay. *Mutat. Res*., 370, 115–120.
28. Baumgartner, A., Cemeli, E. and Anderson, D. (2009) The comet assay in male reproductive toxicology. *Cell Biol. Toxicol*., 25, 81–98.
29. Gutzkow, K. B., Langleite, T. M., Meier, S., Graupner, A., Collins, A. R. and Brunborg, G. (2013) High-throughput comet assay using 96 minigels. *Mutagenesis*, 28, 333–340.
30. Vivek Kumar, P. R., Cheriyan, V. D. and Seshadri, M. (2009) Could a strong alkali deproteinization replace the standard lysis step in alkaline single cell gel electrophoresis (comet) assay (pH>13)? *Mutat. Res*., 678, 65–70.
31. Fatur, T., Lah, T. T. and Filipic, M. (2003) Cadmium inhibits repair of UV-, methyl methanesulfonate- and N-methyl-N-nitrosourea-induced DNA damage in Chinese hamster ovary cells. *Mutat. Res*., 529, 109–116.
32. Gu, L., Gao, W., Yang, H. M., Wang, B. B., Wang, X. N., Xu, J. and Zhang, H. (2016) Control of Trx1 redox state modulates protection against methyl methanesulfonate-induced DNA damage via stabilization of p21. *J. Biochem*., 159, 101–110.
33. Will, O., Gocke, E., Eckert, I., Schulz, I., Pflaum, M., Mahler, H. C. and Epe, B. (1999) Oxidative DNA damage and mutations induced by a polar photosensitizer, Ro19-8022. *Mutat. Res*., 435, 89–101.
34. Richard, M. J., Guiraud, P., Monjo, A. M. and Favier, A. (1992) Development of a simple antioxidant screening assay using human skin fibroblasts. *Free Radic. Res. Commun*., 16, 303–314.
35. Laval, J. (1996) Role of DNA repair enzymes in the cellular resistance to oxidative stress. *Pathol. Biol. (Paris)*., 44, 14–24.
36. Lomax, M. E., Folkes, L. K. and O'Neill, P. (2013) Biological consequences of radiation-induced DNA damage: relevance to radiotherapy. *Clin. Oncol. (R. Coll. Radiol)*., 25, 578–585.
37. Bilbao, C., Ferreiro, J. A., Comendador, M. A. and Sierra, L. M. (2002) Influence of mus201 and mus308 mutations of *Drosophila melanogaster* on the genotoxicity of model chemicals in somatic cells in vivo measured with the comet assay. *Mutat. Res*., 503, 11–19.
38. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA repair and mutagenesis*. First. ASM Press, Washington.