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REVIEW ARTICLE

Comet assay: an essential tool in toxicological research

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Abstract The comet assay is a versatile, reliable, cost-efficient, and fast technique for detecting DNA damage and repair in any tissue. It is useable in almost any cell type and applicable to both eukaryotic and prokaryotic organisms. Instead of highlighting one of the numerous specific aspects of the comet assay, the present review aims at giving an overview about the evolution of this widely applicable method from the first description by Ostling and Johanson to the OECD Guideline 489 for the in vivo mammalian comet assay. In addition, methodical aspects and the influence of critical steps of the assay as well as the evaluation of results and improvements of the method are reviewed. Methodical aspects regarding oxidative DNA damage and repair are also addressed. An overview about the most recent works and relevant cutting-edge reviews based on the comet assay with special regard to, e.g., clinical applications, nanoparticles or environmental risk assessment concludes this review. Taken together, the presented overview raises expectations to further decades of successful applications and enhancements of this excellent method.

Keywords Comet assay · DNA damage · DNA repair · Genotoxicity · Single-cell gel electrophoresis

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Evolution

Over 30 years ago, O. Ostling and K. J. Johanson published the first paper dealing with the single-cell gel electrophoresis assay. They named their technique "microelectrophoresis" (Ostling and Johanson 1984), which some years later was termed "comet assay" (Olive et al. 1990). They embedded mammalian cells (murine lymphoma cell line L5178Y-S and Chinese hamster fibroblast cells, Cl-1) in agarose onto a glass slide and lysed the cells using a neutral detergent solution, and a weak electric field was then applied followed by staining with acridine orange and evaluation in a Leitz MPV2 microscope photometer. The DNA had migrated toward the anode, with more pronounced effects in irradiated than in control cells. In addition, Ostling and Johanson also demonstrated a fast repair process where more than 50 % of the radiation-introduced damages were repaired during 60 min. In conclusion, they postulated a high potential of the method as a predictive test of the efficiency of radio- and chemotherapy of human tumors due to the high sensitivity of the test. At the same time Singh (2016) developed the idea to electrophorese cells in order to move the negatively charged DNA fragments outside of the nucleus and published the paper "A Simple Technique for Quantification of Low Levels of DNA Damage in Individual Cells" (Singh et al. 1988). They used human leukocytes exposed to X-irradiation or H_2O_2. Taking into account that neutral conditions for lysis and electrophoresis do not allow for the detection of single-stranded DNA breaks, they decided to use alkaline conditions. While the migration patterns were relatively homogeneous among cells exposed to X-rays, the effect of H_2O_2 was rather heterogeneous. Furthermore, the repair capacity was completely different between the cells. They described the developed method as a simple approach for the sensitive detection of DNA

damage and repair in individual cells. At least three facts made this method especially attractive in comparison with other equally sensitive methods (Kohn and Grimek-Ewig 1973) for the detection of single-strand breaks: First, only about 1000 cells were required; second, cells did not need to be labeled with a radioisotope, thus allowing the use of any nucleated cell; and third, the method can be used to determine variations in response to DNA-damaging agents between cells of the same population (Olive and Banath 2006).

This alkaline form of the comet assay was rapidly adjusted for use in genotoxicity testing in vitro as well as in vivo. In 1999 an expert panel met to develop guidelines for the use of the single-cell gel/comet assay in genetic toxicology. They reached consensus that the optimal version for identifying agents with genotoxic activity was the alkaline (pH > 13) comet assay developed by Singh and colleagues (Singh et al. 1988). This version allows to detect DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA–DNA/DNA–protein cross-linking, and SSB associated with incomplete excision repair sites. In comparison with other genotoxicity tests, the advantages of the comet assay are: sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells, flexibility, low costs, simplicity of application, and short time needed to complete an experiment. The panel clarified that the comet assay guidelines represent a work in progress (Tice et al. 2000). Eleven years later, the EFSA Scientific Committee reviewed the current state of the science on genotoxicity testing. They recommend a stepwise approach for the generation and evaluation of information on genotoxicity, beginning with a bacterial reverse mutation assay and an in vitro micronucleus assay. In the event of inconclusive or contradictory results, an appropriate in vivo study is recommended. Thereby, the comet assay is one of the suited test systems (Fig. 1) due to its sensitivity to substances which induce gene mutations and/or structural chromosomal aberrations and the usability with many different target tissues (EFSA Scientific Committee 2011). A 2012 published EFSA report summarized the minimum requirements necessary for conducting and reporting results based on the in vivo alkaline comet assay, considering that at this time no OECD Test Guideline existed (European Food Safety Authority 2012). They highlighted that the comet assay can be used to assess the genotoxicity of a great number of chemicals, such as contaminants, pesticides, food contact materials or food additives. Furthermore, the comet assay might help to predict the carcinogenic potential of chemicals (Kang et al. 2013).

The OECD Test Guideline 489 (Fig. 1) for the in vivo mammalian alkaline comet assay was published in autumn 2014 (OECD 2014). It summarizes basics and limitations, principle of the method, verification of laboratory proficiency, historical control data, and a detailed description of the method. After inclusion of the comet assay in OECD 489, a first review which highlights the main technical recommendations was published only a few months later (Araldi et al. 2015). According the OECD, comets should be scored quantitatively with an automated or semiautomated image analysis system. Figure 2 shows typical comet images using the Comet Assay IV™ software of Perceptive Instruments.

Cells should be classified into the three categories scorable, non-scorable, and hedgehog. Only cells with a clearly defined head and tail with no interference with neighboring cells should be scored. The recommended evaluation parameter is % tail DNA or tail intensity (TI), which corresponds to the intensity of the comet tail relative to the total intensity (tail plus head) and reflects the amount of DNA breakage (Kumaravel et al. 2009). An interesting alternative outcome might be the actual DNA break frequency. This can be calculated using a calibration curve based on

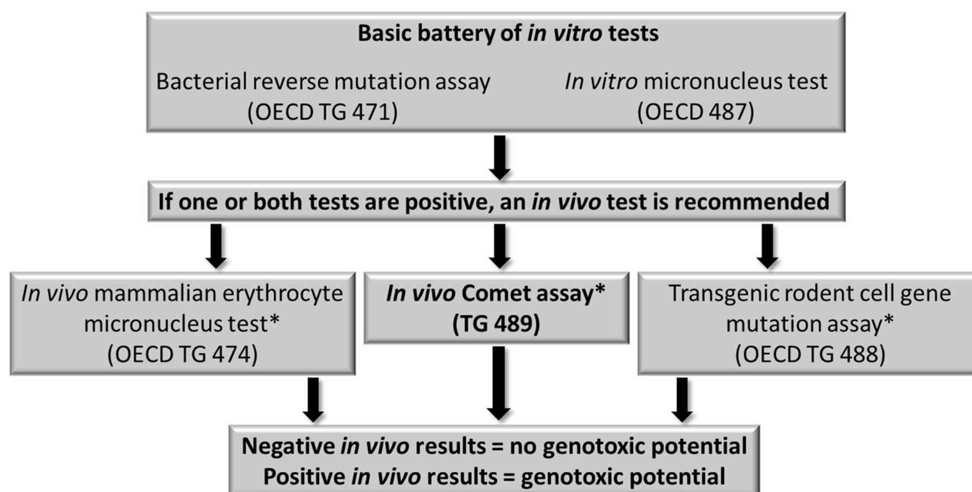

Fig. 1 Comet assay as an integral part of the genotoxicity testing strategy of the EFSA Scientific Committee, *asterisk* in vivo test selected should relate to the genotoxic endpoints identified during in vitro tests According to European Food Safety Authority (2012), OECD (2014)

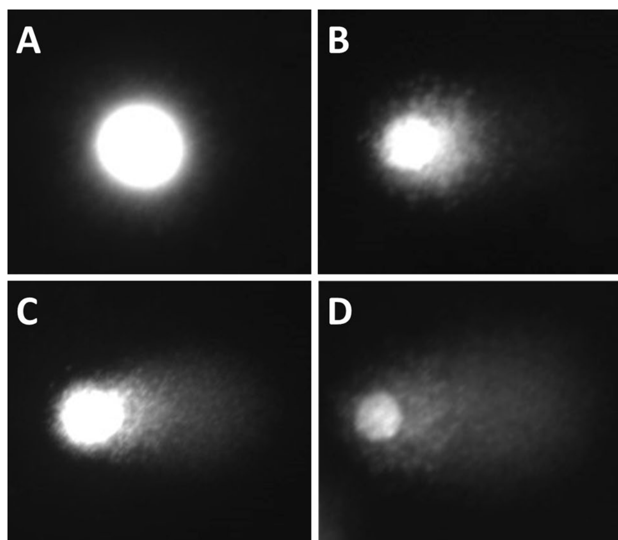

Fig. 2 Comet assay images of HT29 cells after treatment (5 min on ice) with increasing concentrations of H_2O_2 resulting in different stages of DNA damage, **a** 0 µM, undamaged cells; **b** 50 µM, slight damage; **c** 100 µM, increased damage and D) 150 µM, severe damage. Analysis was performed after staining with SYBR® Green using the Perceptive Instruments' Comet Assay IV™ software (magnification: ×40)

an exposure of cells to ionizing radiation as different doses of gamma or X-rays up to 10 Gy lead to a nearly linear relationship between % tail DNA and radiation dose (Collins et al. 2008). Based on the fact that each Gy induces 0.3 breaks per 10^9 Dalton DNA (Ahnström and Erixon 1981), it is possible to transform the TI to breaks per cell, breaks per 10^6 base pairs, or breaks per 10^9 Dalton DNA (Karlsson et al. 2015).

Due to the appearance of hedgehogs (also named ghost cells or clouds), which are considered to be heavily damaged cells (small or nonexisting head, large diffuse tail), TI measurements by image analysis are unreliable. Therefore, such cells should be evaluated separately. Till now the etiology of hedgehogs is rather uncertain (OECD 2014), but comet data using rat liver samples suggest that they can be the result of mechanical-induced damage during sample preparation or substance-related cytotoxicity (Guerard et al. 2014). Furthermore, they were detected using a formamidopyrimidine DNA glycosylase (FPG)-modified comet assay in different tissues of methyl methanesulfonate (MMS)-treated mice, indicating a high level of oxidized and/or alkylated bases and/or FPG lesions (Le Hégarat et al. 2014). In addition, previous studies suggest that they are not diagnostic of apoptosis and should not be taken as an indication of cytotoxicity. As such comets frequently reflect the upper end of a continuum of damage, they should be considered in any overall assessment of genotoxic DNA damage (Lorenzo et al. 2013). The influence of cytotoxic effects on results from the comet assay is a subject of debate until today. So far, at least 70–75 % cell viability should ensure reliable comet assay results (Martins and Costa 2015).

The external scientific report about the genotoxicity of nivalenol (NIV) and deoxynivalenol (DON) is an excellent example of how the comet assay can be used in vivo and in vitro (Le Hégarat et al. 2014). The authors performed the comet assay with and without FPG in seven organs of mice. In addition, to clarify the genotoxic mode of action of both mycotoxins, TK6 cells were used to investigate potential genotoxic oxidative stress induced by the toxins. DON and NIV failed to induce DNA damage in all organs observed and did not induce DNA damage in TK6 cells even after glutathione depletion. Both mycotoxins were classified as free from genotoxic potential.

For those interested in information about the origin and early development of the comet assay, Singh's reflections about the test are highly recommended (Singh 2016). Driven by the idea to create a technique for investigating aging and the extension of lifespan, he developed the method during the last three decades considering different aspects such as cell types (e.g., lymphocytes, germ cells, bacteria), DNA damage trigger (e.g., X-rays, radiofrequency radiation, gamma ray, ethanol, acetaldehyde, vitamin C, diseases, lifestyle factors), or methodical factors (e.g., electrophoresis chamber, slides, analyzing parameters).

Methodical aspects

The comet assay allows the detection of intercellular differences in DNA damage and repair in virtually any eukaryote cell population as wells as in other organisms, such as invertebrates, bacteria, or plants, yeast, and fungi (Bedekar et al. 2014; Dhawan et al. 2009; Frenzilli et al. 2009; Peycheva et al. 2014) on the condition that it can be obtained as a single-cell suspension. On a positive note, with 1–10,000 cells it requires only extremely small cell numbers (Kelvey-Martin et al. 1993). Up to now the comet assay has been successfully used, for example, to measure DNA damage and/or repair in leukocytes (Glei et al. 2002b; Glei and Pool-Zobel 2006), buccal cells (Glei et al. 2005), salivary gland tissue (Ersson et al. 2011), primary colon cells (Glei et al. 2006b), different cancer cell lines like HT29 (Munjal et al. 2012), HT29 clone 19A (Glei et al. 2002a, 2003), LT97 (Glei et al. 2007), or HepG2 (Glei et al. 2006a), and also different epithelial cells (Rojas et al. 2014) like brain cells (Mohamed and Hussien 2016), sperm cells (Cortes-Gutierrez et al. 2014), plant cells (Ventura et al. 2013), yeast cells (Miloshev et al. 2002), or *Drosophila melanogaster* (Gaivao and Sierra 2014).

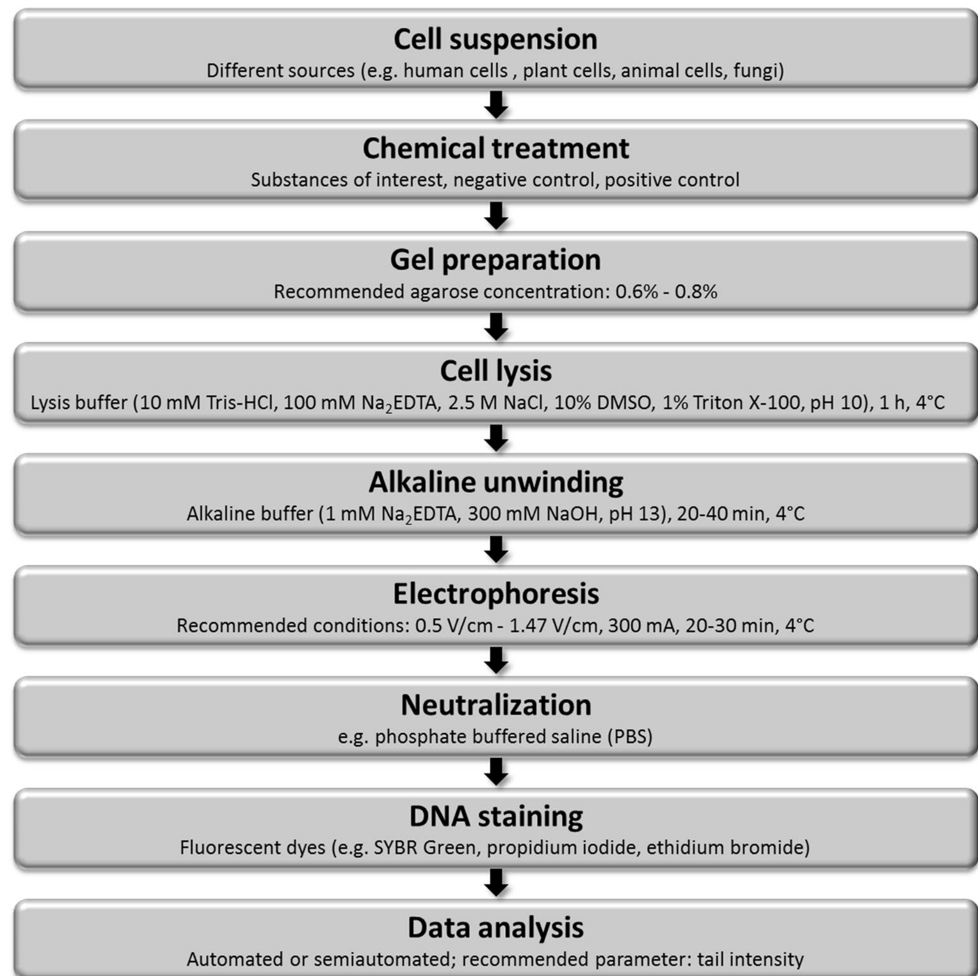

Fig. 3 Basic steps of the alkaline version of the comet assay

Meanwhile the comet assay has achieved a high degree of awareness. This is reflected in nearly 10,000 PubMed hits using the search term "comet assay." These include a large number of general reviews dealing with this test system (Collins et al. 1997b, 2014; Collins and Horvathova 2001; Collins 2009, 2014; Kelvey-Martin et al. 1993; Speit and Hartmann 2005; Tice et al. 2000). Therefore, it does not seem wise and necessary to repeat all the technical details. In particular, beginners are recommended to become informed by considering one of the newer reviews which was also published in this respected journal (Azqueta and Collins 2013). Furthermore, the "Comet Assay Interest Group" (http://www.cometassay.com/) as a free forum for information and exchange is a helpful platform for discussion of all issues related to this test.

Only the main steps of the classic comet assay should be named once more: The cell suspension is mixed with agarose and spread onto a microscope slide, covered with a cover slip, chilled to form a thin gel; slides are placed in a lysis solution (10 mM Tris–HCl, 100 mM Na_2EDTA, 2.5 M NaCl, 10 % DMSO, 1 % Triton X-100, pH 10) for at least 60 min at 4 °C to remove membranes, soluble cell, and nuclear components, leaving DNA attached to the nuclear matrix; slides containing the highly condensed DNA of the former cells, what is called nucleoid, are placed into a cooled electrophoresis chamber containing alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13), for 20–40 min, then electrophoresed for 20–30 min at a voltage gradient of around 1 V/cm; after neutralization the slides will be stained with an appropriate fluorescent dye and measured at a suitable magnification on a fluorescence microscope equipped with specific detectors or a digital camera (Collins 2014; Glei et al. 2003; OECD 2014) (Fig. 3).

Statistical issues in the use of the comet assay were addressed in an earlier work (Lovell and Omori 2008). It seems obvious that the more the cells/comets measured per unit, the more accurate the estimate of the statistics. But, sample sizes of 50 cells per slide are likely satisfactory as the central limit theorem becomes effective when the number of cells exceeds 30.

Following, some exciting or critical aspects of the method/protocol are highlighted as examples.

Influence of basic parameters

Already small changes in comet assay variables may significantly bias the detectable effects of the treatment. Mixed peripheral human lymphocytes from different donors and the human lymphoblast cell line TK-6 were used to examine the influence of agarose concentration, alkaline unwinding and electrophoresis time, as well as voltage and current (Azqueta et al. 2011a). The main outcomes were: (1) Agarose concentration between 0.6 and 0.8 % are optimal, lower concentrations lead to unstable gels, while higher concentrations impede the comet tail generation. The dose–response relationship is significantly linear and damaged cells are more sensitive to the agarose concentration (Ersson and Moller 2011). It is important that the agarose stock solution does not become too concentrated as a result of repeated melting. Therefore, only small aliquots should be stored. (2) Forty minutes for alkaline unwinding is recommended, but shorter times [at least 20 min, (OECD 2014)] are possible. (3) DNA migration is linearly associated with the electrophoresis time and with the potential used. A voltage of 1.15 V/cm applied for a 20-min electrophoresis seems to be optimal. Lower voltage gradients (at $\leq$0.49 V/cm tails are rare) decrease and higher ones (at 1.48 V/cm tails tend to appear detached from heads) increase the TI. It is important to note, that keeping "V/cm $\times$ electrophoresis time" constant will lead to the same DNA migration.

Evaluation

The conventional comet assay format uses 1 or 2 gels on a microscope slide. To increase the throughput different alternatives were developed. For instance, a 3-mm-thick silicon gasket allows to create 12 small gels on one slide, enabling incubation of individual gels with different test substances or enzymes (Shaposhnikov et al. 2010). For large screening studies, a further improvement was recently realized. A Norwegian team has designed and validated a comet assay format with 96 agarose mini-gels supported by a hydrophilic polyester film. This modified, high-throughput (HT) format appears to work with any cell type or tissue and is particularly suited when large numbers of samples need to be processed (Gutzkow et al. 2013). A comparative performance test of standard (2 large gels per slide), medium (12 mini-gels per slide) and HT (24 mini-gels per GelBond® film arranged within a standard 8 $\times$ 12 array) comet assays revealed very similar results when tested with TK-6 cells treated with MMS or X-rays (Azqueta et al. 2013). In addition, the group addressed the so-called edge effect describing the problem that comets with tails at the edges of a gel are not representative of the rest of the gel. They showed that edge distortions are preventable. Therefore, gels should be kept cold at all times and be protected against drying before lysis.

In any case the number or density of cells and comets is an important parameter influencing the efficiency and reliability of analysis and evaluation. It should not be so many that they overlap, as this makes scoring challenging or impossible, and if the density is too low, it is time-consuming to locate the cells. Collins group recommended placing a few thousand cells in a conventional large gel, or only a few hundred in a mini gel. Important to add, all samples should be adjusted to a defined cell concentration. This means that a fixed volume of cell suspension has to be added to a fixed volume of agarose to achieve the required cell density (Collins 2014). Meanwhile, a comprehensive overview considering the most relevant HT comet assay systems for the last 15 years has been published (Brunborg et al. 2014). Some of the HT systems rely on cutting-edge technology, whereas others are low-cost modifications of the original method. It is expected that advanced methodologies such as the recently developed CometChip Technology which uses microfabrication technology to produce a microarray of precisely ordered microwells within a bed of agarose, each of them with a configurable diameter as small as a single cell (Watson et al. 2014) or the agarose-based micofluidic chip (100 parallel microchannels, 20 $\times$ 20 µM) capable of simultaneously interrogating DNA damage information of 10,000 individual cells (Li et al. 2013) will be increasingly used in near future.

The scoring of DNA distribution/comets remains the time-limiting step of the comet assay. To verify the suitability of different methods the performance of visual scoring, semiautomated and automated image analysis were compared (Azqueta et al. 2011b). For this, human lymphoblastoid TK-6 cells treated with MMS (0.04–0.6 mM) and H_2O_2-treated peripheral lymphocytes (2.5–160 µM) were used and comets in the same set of slides were differently measured. During visual scoring, 50 comets on each gel were classified as belonging to one of five damage categories in accordance with head and TI as described earlier in more detail (Collins et al. 1997a). For this, a Nikon Eclipse TS-100 fluorescence microscope was used. In combination with an image analysis system (Comet Assay IV, Perceptive Instruments), a semiautomated evaluation was possible. Here, 50 comets on each gel selected by the operator were analyzed, and the percentage of DNA in the tail was used for evaluation. Although in principle possible, the option to change the program defined set of parameters (e.g., beginning of head or end of tail) was not used during the experiments. The automated image analysis system (Pathfinder™ Cellscan Comet system) detects comets in boundless numbers without manual intervention. Therefore, the number of analyzed comets varied from gel to gel. In summary, the authors stated that visual scoring is cheap and simple, but shows some deviation from linearity regarding break frequency; semiautomated and automated scoring

are particularly suited for experiments with a high number of samples. However, the most important result from this investigation is that findings from all three approaches may be considered to be trustworthy and interchangeable. Nevertheless, meanwhile new guidelines recommend the use of semiautomated and automated scoring systems in order to enhance standardization of the method (OECD 2014).

Effect of cell cycle

Although cells actively replicating their DNA seem to react differently during gel electrophoresis depending on pH [alkaline conditions: S-phase DNA migrates more rapidly because the replication forks act like SSBs; neutral conditions: S-phase DNA operates as replication bubbles that slow down migration (Olive 1999)], it is possible to measure damage in any phase of the cell cycle, because the assay considers both DNA content and DNA damage (Olive and Banath 2006). This was proofed in unpublished experiments comparing the effect of X-ray radiation using synchronized cells and cells with different cell cycle stages. Consequently, the comet assay is suitable for the use with asynchronous cultured cells as well as for primary cells where the cell cycle stage is not controllable (Kelvey-Martin et al. 1993).

Oxidative damage

Oxidative stress, defined as an excessive load of reactive oxygen species (ROS) which cause reversible or persistent damage on cellular or systemic level, has linked to disease development and accelerated aging for a long time. Meanwhile, there is growing evidence that the transient increase in ROS levels may allow organisms to protect itself more efficiently from exogenous or endogenous stressors (Ristow 2014). Furthermore, the use of antioxidants is characterized as useless or even harmful (Ristow and Schmeisser 2014). A so far unanswered question is how transient oxidative stress could be reflected by oxidative DNA damage and its repair. This might be measured by using the enzyme-modified comet assays. This method was already often described in detail (Collins et al. 1993; Collins and Horvathova 2001; Collins 2009, 2014). The crucial step toward detecting of oxidized bases is the incubation of the nucleoids after lysis with lesion-specific repair endonucleases, e.g., FPG, recognizing altered purine bases including 8-oxo-guanine, or endonuclease III, which responds to oxidized pyrimidines (Glei et al. 2005; Glei and Pool-Zobel 2006). The enzymes induce additional breaks at the sites of oxidized bases and increase the DNA in the tail of the comets. Here, it must be ensured that the duration of incubation with the repair enzymes affects the DNA migration. Using A549 lung carcinoma cells treated with the photosensitizer Ro 19-8022 and visible light [generates mainly 8-oxo-guanine (Will et al. 1999)], it was possible to demonstrate that the DNA migration is significantly increased when treatment with FPG was done for 30 or 45 min in comparison with 10 min (Ersson and Moller 2011). In contrast to the above-mentioned potential harmful effects of antioxidants on health parameters, studies dealing with the influence of antioxidants on oxidative DNA damage reveal a somewhat different picture. While a 12-week intervention with different single carotenoids has failed to have a significant effect on endogenous oxidative damage in lymphocytes of healthy non-smokers (but, high levels of damage were not seen) (Collins et al. 1998), a mixture of vitamin C, vitamin E, and β-carotene resulted in a highly significant decline in endogenous oxidative base damage in lymphocytes of non-smokers and smokers (Duthie et al. 1996). In addition, the initial higher level of oxidized bases of smokers had disappeared after 20 weeks of supplementation. Not only the kind of antioxidants but also the formulation influences potential effects as shown for slow release and plain release vitamin C formulations. In male smokers, only the ingestion of slow release vitamin C led to fewer FPG- and endonuclease III-sensitive sites (Moller et al. 2004). Moreover, in combination with the determination of genetic polymorphisms in relevant genes, the comet assay can provide information on associations between the genetic background and environmental factors. Here, it should be noted that the necessary sample size for the biomarker approaches will expand taking into account the needs of polymorphism analysis. Significant effects on phenotype might be seen in a group of 50 people when considering a common genotypic variation, dealing with less common variants far larger numbers are necessary. The case numbers further increase when interactions between polymorphisms are considered (Collins 2009). The stratification of 38 healthy male volunteers of an intervention study into subjects with *GSTM1*1* and *GSTM1*0* genotypes revealed no differences for strand breaks, whereas at the baseline oxidized bases tended to be higher in *GSTM1*0* than in *GSTM1*1* in smokers and non-smokers. A statistically significant intervention effect (bread with antioxidative supplements) was apparent only in the *GSTM1*0* smokers (Glei et al. 2005). Polymorphisms in repair genes are of special interest. Some excision repair gene polymorphisms modify the susceptibility to bladder cancer. For example, polymorphisms (codons 312 and 751) in xeroderma pigmentosum group D (*XPD*) gene increase cancer risk, whereas a combination of homozygous wild-type genotypes were associated with a twice lower frequency in $T \geq 2$ carcinomas suggesting that the maintenance of normal DNA repair activity seems to inhibit cancer initiation and/or cancer progression (Savina et al. 2016).

DNA repair

The physicochemical constitution of the DNA is unable to guarantee lifelong stability and correct function. The main causes of DNA lesions with important implications for mutations and potential carcinogenetic processes are environmental agents (ultraviolet component of sunlight, genotoxic chemicals, ionizing radiation), products of normal cellular metabolism (ROS derived from oxidative respiration, lipid peroxidation products), and the instability of some chemical bonds in DNA (tendency to spontaneously disintegration). The molecular machinery evolutionary designed to counteract the detrimental genetic degeneration is vital. It includes base excision repair (BER) dealing with abasic sites, 8-oxoguanine, or SSB; nucleotide-excision repair (NER) taking care of bulky adducts or cyclobutane pyrimidine dimers; recombinational repair for double-strand breaks and interstrand cross-links; and mismatch repair dealing with base mismatches, insertions, or deletions (Hoeijmakers 2009, 2001). Cells respond to DNA lesions by activating complex signaling networks that decide about cell fate, promoting not only cell death but also DNA repair and survival (Roos et al. 2016). Due to these repair mechanisms the level of DNA damage remains largely constant, at least in healthy people. Our knowledge about the complex DNA repair processes grows rapidly. Examples include the cutting-edge reviews about DNA double-strand-break repair in higher eukaryotes and its relevance in genomic instability and risk of cancer (Mladenov et al. 2016), or the BER as a pathway which is mainly regulated by posttranslational modifications (Carter and Parsons 2016). The DNA repair capacity is considered as a valuable marker of susceptibility to mutation and cancer, which means that a suboptimal repair activity is associated with a higher risk of, e.g., squamous cell carcinoma of head and neck (Liu et al. 2016b). The repair potential is frequently determined at the level of transcription by using DNA microarray or RT-PCR for genes involved in the DNA repair pathways. But, the activity of enzymes does not just depend on the rate of transcription or translation, so a phenotype assay seems to be a better choice (Azqueta et al. 2014). There are at least three current reviews available dealing with the comet assay as a suitable method to phenotypically reflect DNA repair processes (Azqueta and Collins 2013; Azqueta et al. 2014; Collins 2014). Therefore, only some selected aspects shall be highlighted without considering technical details.

The simplest way to detect kinetics of repair processes is to perform the comet assay on cells at different times after treatment with DNA-damaging agents, meanwhile called as challenge assay (Au et al. 2010). This was done, e.g., with human lymphocytes incubated with water-soluble β-carotene or lycopene and then treated with bleomycin or H_2O_2. The results indicated that β-carotene protects against strand breaks but not against oxidized bases, and did not modulate repair of bleomycin- or H_2O_2-induced DNA damage (Glei et al. 2002b). In contrast, β-cryptoxanthin protected HeLa- and Caco-2-cells from damage induced by H_2O_2 and showed a striking effect on DNA repair. This carotenoid led to a doubling of the rejoining rate of strand breaks and the rate of removal of oxidized purines (Lorenzo et al. 2009). Thereby, the standard comet assay is used to determine the capacity of cells to rejoin breaks. If lesion-specific enzymes are used, as mentioned above, the elimination of a particular type of damage can be evaluated (Azqueta et al. 2014). That means the specificity of the assay is determined by the kind of the DNA-damaging agent and the type of enzyme used. In biomonitoring studies H_2O_2 or radiation is commonly used to damage the DNA of lymphocytes from cancer cases and controls, and SB rejoining is examined. But, differences can reflect a cause or an effect of the disease. An interpretation in terms of cancer susceptibility seems not to be reasonable (Collins and Azqueta 2012). Nevertheless, accumulation of oxidatively induced DNA damage might serve as a potential biomarker of genome instability predisposing to cancer as recently shown by comparing the damage response in H_2O_2-treated lymphocytes using the comet assay in bladder cancer patients as compared to healthy controls, elderly persons, and individuals with inflammations (Savina et al. 2016).

One theoretical aspect relating to the challenge assay for DNA repair has to be added. Frequently, the residual damage is measured at only one or a small number of time points after the treatment. But, to generate reliable information different times of measurement are useful, and ideally repair should be detected at shorter intervals immediately after treatment, since the initial rate of removal or the $t_{1/2}$ are stronger parameters to study (Collins and Azqueta 2012). Using this approach, it was possible to show that $t_{1/2}$ for rejoining of strand breaks was about 10 min for HeLa and 18 min for Caco-2 cells. Repair of oxidized bases lasted much longer with $t_{1/2}$ of about 135 and 260 min in HeLa and Caco-2 cells, respectively (Lorenzo et al. 2009). The highest repair activity of mutagen-exposed phytohaemagglutinin (PHA)-stimulated lymphocytes was measured at the beginning of the culture (e.g., 1 h after MMS exposure) and repair continuously decreased in the course of cultivation (Bausinger and Speit 2015).

Some modified versions of the challenge assay were developed. A combination of the comet assay with the fluorescent in situ hybridization, the Comet-FISH (Glei et al. 2009; Glei and Schlörmann 2014), has been used to study DNA damage (Glei et al. 2007) and DNA repair (Shaposhnikov et al. 2011) in selected genes or particular DNA sequences. This assay allows monitoring the DNA

damage repair of a specific gene by following the migration of gene-specific signals from the comet tail into the comet head in the course of time, as shown for the human tumor suppressor gene p53 in peripheral blood mononucleated cells (PBMCs) (Horvathova et al. 2004). The use of specific repair inhibitors can help to identify the pathways involved in damage repair or to make the assay more sensitive by preventing repair synthesis. A 2010 published study describes the development of a cellular phenotype assay for NER (by using a NER-deficient fibroblast cell line), based on the use of benzo[*a*]pyrene diol epoxide (BPDE) as model mutagen (Vande Loock et al. 2010). After in vitro challenge of PBMCs with BPDE and the use of the DNA polymerase inhibitor aphidicolin (APC), it was possible to discriminate between both types of breaks, strand breaks resulting from direct interaction with DNA and incisions introduced by repair enzymes. Applying the assay to PBMCs from 22 donors revealed a higher inter-individual variation of the repair capacity in comparison with the intra-individual variation. A further study combined the comet assay with the repair inhibitor APC and array gene expression analysis of 92 DNA repair genes (Bausinger and Speit 2015). The researchers examined the repair of DNA lesions induced by BPDE and MMS in PHA-stimulated human lymphocytes especially in the period before replication. The data indicate that the removal of BPDE-induced damages was slower than the repair of MMS-induced lesions. BPDE led to altered expression of several genes, but only two genes (*XPA*, *XPC*) were directly related to NER. Among the 15 genes specifically associated with NER, only *XPC* was enhanced in expression (≥twofold) after treatment with BPDE under all experimental conditions tested. The results showed that lymphocytes repair mutagen-induced excisable DNA lesions in the course of cultivation before they enter the S-phase, with the highest repair activity immediately after exposure.

A more sophisticated or biochemical approach to measure DNA repair than the challenge assay, especially in cases where many samples have to be processed at the same time [e.g., during biomonitoring studies (Collins and Azqueta 2012)] or to overcome some theoretical problems of the challenge assay (Azqueta and Collins 2013), is the in vitro DNA repair assay. In this method a cell-free extract containing a certain amount of repair enzymes (usually from PBMCs) is incubated with agarose-embedded nucleoids including a specific lesion derived by lysis of cells that have been incubated with suitable DNA-damaging substances. DNA breaks accumulating during the incubation are used to monitor the repair capacities of the cell extracts. Different schemes of the comet-based in vitro DNA repair assay can be found in current publications, e.g., one illustrates the assay for BER (Azqueta and Collins 2013), and another is more general (Azqueta et al. 2014). The authors note that nucleoids have to contain an excess of lesions for the cell-free extract to work and unwanted damages should be low. To be able to differentiate levels of repair activity between different extracts the time of incubation should be critically chosen. Furthermore, appropriate non-damaged control nucleoids are required to consider the activity of non-specific nucleases (Gorniak et al. 2013). The use of the in vitro DNA repair assay as a suited biomarker in human biomonitoring studies considering influences of occupation, environmental, or lifestyle factors as well as of repair gene polymorphisms (Collins and Azqueta 2012), and the test application not only in cell culture and animal studies, but also in human, occupational, and nutritional studies (Azqueta et al. 2014), were recently reviewed. In addition, the assay was adjusted to measure BER- and NER-specific DNA repair capacity in tissues of different transformation stages using seventy pairs of tumor and adjacent healthy colon tissue samples (Slyskova et al. 2012). The analysis revealed that colon tumor cells are not deficient in BER and NER, but rather show individual characteristics.

In any comparative investigation with the in vitro DNA repair assay, it is essential to start with the same number of cells in each extract. The detectable repair activity is reliant on the protein concentration in the extract but is not directly proportional. Adjustment of measured activities against protein concentration is of doubtful accuracy (Azqueta and Collins 2013).

Interpretation of results

There is no direct relationship between the amount of DNA damage induced by different chemicals or radiations and the biological importance of the damage. One complicating factor is that chemicals that induce cross-links (DNA–DNA or DNA–protein) will counteract the detection of single-strand breaks (Speit and Hartmann 2005). For this reason, investigation of chemical mixtures or substances with different modes of action can be very complicated. Therefore, comparing comet assay results with data from other test systems (e.g., chromosome aberrations, adduct levels, micronuclei) to interpret the biological relevance is highly recommended (Olive and Banath 2006) and is an inherent part of genotoxicological test strategies (EFSA Scientific Committee 2011).

Current work

The comet assay has become a method used around the world for better monitoring and comprehension of DNA damage. This is reflected by numerous and growing numbers of publications every year dealing with quite different scientific problems. Figure 4a represents the growing

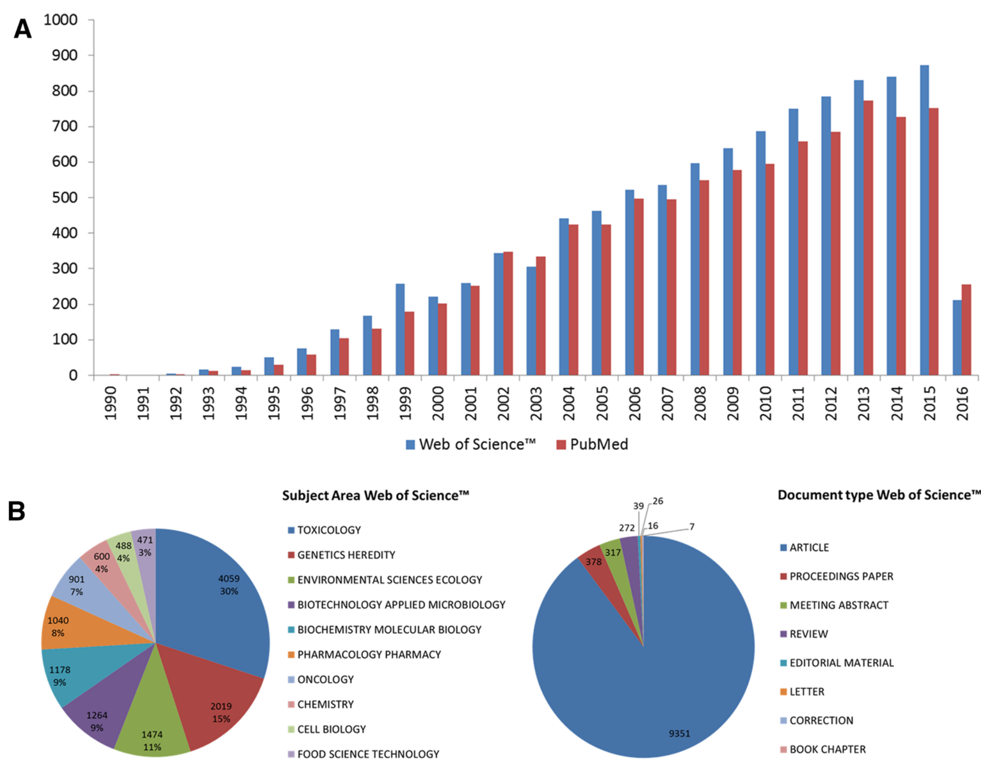

Fig. 4 Publications based on the comet assay in PubMed- and Web of Science™-indexed journals from 1990 to 2016 (including publications until April 2016). **a** Overview of the growing number of publications per year. **b** Overview of the subject area and document types revealed by the search in Web of Science™ Core Collection. Results were drawn from the PubMed (total counts: 9091) and Web of Science™ Core Collection (total counts: 10,037) databases including the years from 1990 to 2016 using the search term "comet assay"

number of publications considering the comet assay during the last 26 years. Since then, about 10,000 articles, including about 270 reviews, have been published using the comet assay. According to the Web of Science™ Core Collection database, these published articles mainly belong to toxicological research areas which account for 30 % of all subject areas using the comet assay. But this method is not limited to a distinct research area but rather applicable in a widespread field of subject areas like genetics, environmental science, and biotechnology as shown in Fig. 4b.

To understand for what the comet assay is used for, the following Tables 1.A and 1.B in Supplementary Material summarize selected information about the 179 latest publications (accessible original work) dealing with the use of this universally applicable method. These were obtained by a PubMed database search including publications from January until April 2016 using the search term "comet assay."

The current work can be almost equally divided into in vitro (Table 1.A of Supplementary Material) and in vivo studies (Table 1.B of Supplementary Material). Their classification to distinct subject areas is comparable to that identified for the publications based on the comet assay method from the last 26 years given in Fig. 4 showing the versatile application of this method. The biggest part of these publications, comprising about 33 %, can be categorized to a research area dealing with toxicological topics such as the genotoxicity assessment of acrylamide and glycidyl methacrylate (Dobrovolsky et al. 2016), followed by 31 % which address specific environmental or ecotoxicological topics such as the assessment of the genotoxic potential of water along the Danube River (Kolarevic et al. 2016). The remaining publications can be classified into subject areas like medicine, cancer research, nutrition, or biochemistry. From all these publications, 67 of the in vitro studies (about 74 %) and 21 of the in vivo studies (about 24 %), respectively, used tissue or cells from human origin, which makes this the most used species, especially in the in vitro comet assay studies. In addition, the in vivo studies also used cells obtained from a numerous different species including mainly rat, fish, mouse and worm. Some few studies also investigated DNA damage in bacteria (Danevcic et al. 2016), plant cells (Cetinkaya et al. 2016; Faisal et al. 2016; Lanier et al. 2015), amoebae (Kusrini et al. 2016) or hydra (Zeeshan et al. 2016). Whereas in the in vitro studies, a wide range of cell types belonging to different organ systems (e.g., A549, HCT 116, HEPG2, MCF-7, MRC-5, BEAS-2B, CHO-K1, and HEK293) were used, the in vivo studies were mainly based on cells from the blood system (about 61 %) like erythrocytes obtained from, e.g., fish or lymphocytes from, e.g., humans. This proves that the comet assay is suitable for almost any tissue or cell type.

Due to its easy handling, a combined examination of genotoxic and antigenotoxic effects for the characterization of new or unknown substances like, e.g., extracts from plants is another advantage of the comet assay. But only a few studies also addressed antigenotoxic effects (~6 %) like, e.g., Sassi et al. (2016) who examined genotoxic as

well as antigenotoxic effects of *Ceratonia siliqua* extracts and total oligomer flavonoids in murine leukemia cells. In contrast, most of the publications investigated genotoxic effects of distinct substances like diazinon (Ezzi et al. 2016b) or nano particles such as Ni nanoparticles (Magaye et al. 2016). A limited number of studies (about 8 %) also analyzed oxidative-induced DNA damage using lesion-specific enzymes like FPG, Endo III or hOGG1 or DNA repair (about 7 %) in combination with the comet assay. The latter was especially examined using the challenge assay.

Most of the current publications (about 51 %) used the TI as main analysis parameter or unit for the degree of the observed DNA damage, but the olive tail moment (OTM), tail moment (TM), and tail length (TL) were also frequently used. The scoring or analysis of the comet assay results was mostly performed using analysis systems such as Comet Assay II–IV, Komet 3.0–7.0, Comet Score, or CASP. Though the comet assay is a sophisticated method, the OECD guidelines recommend a quantitative scoring of the comets using a semiautomated or automated image analysis system (OECD 2014). Therefore, there are still many studies (about 21 % of the current publications) which performed visual scoring by differentiating between comets and no comets or by classification into different damage categories as described earlier (Collins et al. 1997a). Furthermore, even 12 % of the current work gives no information about the method of comet scoring at all.

Surprisingly, a great part of the studies do not mention the use of an appropriate internal comet assay control (e.g., H_2O_2 or ethyl methanesulfonate, EMS), which is necessary to verify the obtained results. There are also large variations regarding the methodical aspects between the different studies, e.g., the duration of the unwinding step ranged from 2.5 to 60 min and electrophoresis was performed in the range of 5 to 90 min. These discrepancies may partly arise from different methodological needs for different cell types. In some studies, detailed information about the critical methodical comet assay steps is missing.

The statistical evaluation of the comet assay results was performed differentially in the current publications, but the ANOVA, followed by Mann–Whitney *U* test, Kruskal–Wallis test and Student's *t* test were the most used tests described.

Taken together, the overview of the latest publications shows that the comet assay is a widely applicable method addressing different subject areas as well as different endpoints using a wide range of organisms or cell types, respectively. But it also demonstrates the differences in methodical aspects especially regarding the evaluation and scoring of comets. Though most of the studies performed the assay according to the OECD guidelines, the overview over the current publications demonstrates that there is still a need to further strengthen the standardization of comet assay protocols.

Current reviews

Every year a number of outstanding reviews dealing with the comet assay are published. Some of the recent ones reflecting the wide field of application are briefly presented in the following.

Clinical applications and biomonitoring

Gunasekarana et al. (2015) describe clinical applications of the comet assay and highlight its potential to support human biomonitoring studies, to improve our understanding of pathogenesis of cancer and progression of chronic and degenerative diseases, as well as to enhance the prediction of tumor radio- and chemosensitivity, and to better recognize male infertility. Due to an abundant number of potential confounding factors of comet assay (some are discussed above) they recommend a further experimental validation and standardization of the method. But, they are convinced that a standardized protocol and analysis system considering different variants of comet assay will be a useful and reliable clinical tool in the field of medicine for the assessment of DNA damage and repair capacities.

Already one year before, the ComNet group (comprising almost 100 research groups) provided an overview of comet assay in general and as a widely used method in human biomonitoring to investigate DNA response to genotoxic and genoprotective agents (Collins et al. 2014). Based on 170 publications, including some comprehensive former reviews (Collins 2009; Collins and Azqueta 2012; Dusinska and Collins 2008; Hagmar et al. 1998; Valverde and Rojas 2009), they describe the diverse applications of the comet assay in different kinds of human studies with particular focus on biomonitoring of environmental and occupational exposures as well as effects of phytochemicals in nutritional intervention studies, and explain factors that are responsible for individual biological variability and influence the susceptibility to cancer and other diseases. Most of these investigations are based on the use of either whole blood or the fraction of leukocytes isolated by density gradient centrifugation. The use of epithelial cells is an exciting alternative for human biomonitoring studies assessing DNA damage as recently also reviewed (Rojas et al. 2014). This report summarizes the so far widely accepted guidelines for the comet assay in different types of epithelial cells, with particular focus on lens cells, corneal cells, exfoliated tear duct cells, buccal cells, and nasal cells (including 3D mini organ cultures of human inferior turbinate epithelia). Lens and corneal cells are used with clear clinical applications; all the others are suited as genotoxicity biomarkers in human monitoring. The comparison of existing methods reveals that a unified protocol for all kind of epithelial cells is hardly possible. But, based on

a specific sampling procedure, the alkaline version of the comet assay is generally recommended. For any cell type, essential notes on slide preparation, lysis, unwinding, and electrophoresis, as well as neutralization, are given. In any case 50 cells/comets should be evaluated per slide. The authors summarize that at present, the comet assay in epithelial cells has been little used. In future, its application could be an excellent tool not only for biomonitoring studies and risk assessment, but also for diagnosis of diseases and treatments.

Nanoparticle-induced genotoxicity

Due to the fact that engineered nanoparticles (NP) have diverse unique properties they are increasingly used in almost all areas, like agriculture, food industry, or medicine (Tekiner et al. 2015; Uthaman et al. 2015; Viswanathan and Manisankar 2015; Wang et al. 2016c), but their possible impact on human health have not yet been adequate studied. Therefore, there is a strong need for more information about potential adverse health effects. One major aspect that has to be considered is the potential of NP to induce DNA damage as shown for instance for diesel and gasoline emission in traffic exhausts (DeMarini 2013) or for engineered nanomaterials (Moller et al. 2015). Golbamaki et al. (2015) analyzed the peer reviewed publications of the nearly last two decades and found that the method most used to evaluate the genotoxic potential of NP is the comet assay, followed by micronucleus, Ames and chromosome aberration tests. Their review is focused on the potential of metal oxide and silica NP to cause genotoxicity and found some inconsistent results for the same core chemical composition. The following reasons may be responsible for conflicting results: various sizes and size distribution of NP used, different purities of NP, varying surface areas, diverse coatings, variations in crystal structure of the same type of NP, different sizes of aggregates in solutions, various assay protocols, and different concentrations used. As both physical and chemical properties can affect NP behavior and may have an influence on genotoxicity, they must be a substantial part of genotoxicity testing (Magdolenova et al. 2014). Underlying mechanisms of interactions between the comet assay and NP were critically discussed in the same year (Karlsson et al. 2015). Since detecting of strand breaks and alkali-labile sites as well as oxidative DNA damage after NP treatment has been mainly employed in nanotoxicology studies to date, this publication focuses on these applications. The main outcomes of evaluating potential interferences between different steps of the comet assay and NP which could result in false positive or false negative results are: an interaction that significantly affects results of the comet assay is unlikely for most NP; exposure to UV light of photocatalytic active NP (e.g., TiO_2) can increase DNA damage; interferences between DNA stains and NP are not excludable, but without particular relevance; it seems that the presence of NP with the nucleoids does not affect the DNA migration; and a selection of NP (e.g., SiO_2, TiO_2, Fe_2O_3) does not impair the activity of the repair enzyme FPG. The authors highlighted a strong consistency between results of comet and micronucleus assay for different NP and concluded that both tests can be trusted in the valuation of NP genotoxicity.

Comet assay in insects

About 15 years ago, the comet assay has been adapted to use it in *Drosophila melanogaster* (Bilbao et al. 2002). In the meantime some other insects were also used as recently reviewed (Augustyniak et al. 2016). They belong to four orders, *diptera* (e.g., *Drosophila melanogaster*), *lepidoptera* (e.g., *plodia interpunctella*), *coleopteran* (e.g., *tenebrio molitor*), and *orthoptera* (e.g., *chorthippus brunneus*). Insects in general are an interesting subject especially of ecotoxicological investigations due to their wide distribution in our ecosystem. *Drosophila melanogaster* is regarded as model organism that is outstanding suited for genetic studies. In seems to be one of the most widely used insects in developmental and genetic research. Five procedures are used to obtain cell suspensions from insect's tissue, spontaneous separation of cells, tissue homogenization in phosphate-buffered saline, fast-freezing in liquid nitrogen, incubation with collagenase, and macerating by squeezing through gauze. The most used cell types are brain cells, hemocytes, midgut cells, imaginal disk cells, and spermatocytes as recently reviewed (Gaivao and Sierra 2014). However, using insect cells in comet assay requires the consideration of some test modifications (Augustyniak et al. 2016). These include the use of low melting agarose at a higher percentage of 1.5 % for *D. melanogaster* cells (considering the smaller size of the cells), reduced unwinding and electrophoresis time (10 and 15 min, respectively), or the use of a lysis solution without dimethyl sulfoxide.

D. melanogaster is a well-established and accepted model for toxicological research and human diseases. Therefore, all the findings of in vivo genotoxicity studies with this insect should be considered as relevant for human beings (Gaivao and Sierra 2014). Presupposed the further standardization and validation of protocols used so far succeed, it is expected that the comet assay will be more used in environmental risk assessment and will help to improve our understanding of phenomena of insect life.

Environmental risk assessment

Environmental risk assessment (ERA) is a strategy that aims to determine if an environmental contamination

exceeds a threshold and causes harmful effects to the resident communities (Chapman 2007). Based on this definition, the state of the art of the comet assay application to marine or brackish water organisms with regard to ERA was reviewed (Martins and Costa 2015). The authors emphasize that the comet assay still holds a lot of constraints in ERA, in large part due to problems in obtaining clear cause–effect relationships from complex environments. This is particularly true if non-model organisms are used. This caused constraints to toxicologists concerning lack of previous biomarker validation, raised intraspecific variability and absent or diminished genomic annotations. Furthermore, considering wild organisms for ERA, the relative sensitivity to contaminants may become an issue; since negative results do not imply that there is no burden. The particular species used could not be exposed to or do not react sensitive to the pollutants. Nevertheless, the alkaline comet assay is applied on sentinel organisms, wild or used in bioassays *in* or ex situ. In addition, there are important efforts to standardize protocols and to establish guidelines to the interpretation of results.

The same is true for the application of the comet assay in the whole field of ecotoxicology as recently also reviewed (de Lapuente et al. 2015). The authors present a comprehensive overview considering the most relevant experimental models (amphibians, e.g., *Xenopus laevis*, *Lithobates clamitans*, which belong to the most sensitive organisms regarding environmental changes; fishes, about 90 different species; mollusks, e.g., bivalves, cephalopods, gastropods; terrestrial organisms, e.g., birds and mammals, earthworms, vegetal cells), the advancement and important modifications of the different protocols and cell types used, and existing correlations with other biomarkers (e.g., micronuclei, products of lipid peroxidation, antioxidant capability, apoptosis, age, gender, or egg production). In addition, needs for further protocol improvements are highlighted. Here it must be ensured that each organism and each case study required their own set of technical features and interpretations, in particular considering non-model native species. Therefore, the clear and conclusive demonstration of its ecological relevance might be the greatest challenge to comet assay during the next years.

A few years ago, it was noted that the use of comet assay in plants is still limited, compared to animal systems (Ventura et al. 2013). But, this method is meanwhile emerging as a useful tool in getting information on genotoxicity of environmental pollution. This is reflected by the fact that at least three current reviews are available covering this topic. In spite of similarities with other eukaryotic models, the comet assay protocols for plant cells must consider important differences, first of all the presence of a rigid cell wall. Recently, the key factors affecting comet assay performance and possibilities to improve its significance were identified (Pourrut et al. 2015). Using four different plant species crucial steps of the method were evaluated. Results indicated that short chopping is more efficient to isolate nuclei than the slicing method. Filtration and lysis steps can be skipped. Furthermore, they showed that light and high room temperatures are able to induce DNA damage in isolated plant cell nuclei. Calibration tests revealed that a special attention should be paid to exposure time, plant growing stage, and leaf position. Santos et al. (2015) reviewed the data from the last 5 years on the use of the comet assay as a standard method in plant ecotoxicological studies. The considered stress conditions are radiation (light, UV, γ-ray, and X-ray), metals (Cd, Zn, Cu, Co, Pb, B, Al, Cr, and As), nanocompounds (metal and metal oxide nanoparticles, quantum dots), organic pollutants (e.g., dyes and pesticides), contaminated matrices (e.g., fly ash, effluents, leachates, or gases), and others. Furthermore, as mutagenic controls EMS, MMS, or N-methyl-N-nitroso-urea (MNU) were used. In summary the authors stated that the recent advances in the use of the comet assay in plants to both a larger number of conditions and an increasing number of plant species demonstrates the suitability of the comet assay to assess DNA damage induced by quite different stress conditions. In addition, the data presented support that this technique may be a useful tool to complement conventional and -omics methods in situ environmental pollution monitoring. In consideration of 101 key publications which describe the use of comet assay in higher terrestrial plant models, it became clear that general consensus validates the use of the alkaline version of the test, the use of percentage of DNA in tail for measuring effects, and use of preferred roots to study (Lanier et al. 2015). According to the collected data, 45 terrestrial higher plant species have been used for comet assay studies. The three most frequent ones were *Allium cepa*, *Nicotiana tabacum*, and *Vicia faba*. It seems that only a few changes to biomass sampling and electrophoretic migration parameters are sufficient to adjust the assay to another species. Roots and bulbs were the major used parts of plants, followed by leaves. As already mentioned above, cell lysis seems not to be essential, and DNA damage can be quantified with and without this step. Most groups rely on computerized image analysis systems and read 75–150 cells per condition. They use just one or two parameters to describe the effects. The three main parameters, all characterizing the comet tail, are TM, TI, and TL. A critical point is that the use of a positive control is not a general rule so far. Altogether Lanier et al. are convinced that the comet assay could be considered a valuable tool for screening the mutagenic potential of environmental samples, although the measured genotoxic effects cannot be extrapolated directly to mutagenicity and carcinogenicity in humans.

Comet assay in mammalian sperm cells

Spermatozoa nuclear DNA damage is associated with infertility (Castilla et al. 2010). To assess DNA damage in sperm cells different methods (e.g., sperm chromatin structure assay) have been developed and modified versions of the comet assay exist, too. The potential of the so-called two-tailed comet assay (two-dimensional perpendicular tail comet assay, TT-comet), suited to differentiate between SSBs and DSBs on the same sperm cell, was recently reviewed (Cortes-Gutierrez et al. 2014). The response of gametic chromatin and somatic DNA to comparable treatments varies dramatically due to the different levels of tissue dependent heterochromatinization and the histone replacement by protamines during spermatogenesis. Interestingly, each species has a different protamine amino acid composition (Vilfan et al. 2004), so that lysing conditions used to induce a controlled protein depletion has to be species specific to make results comparable. Furthermore, due to inherent characteristics of sperm DNA structures of different species, it is necessary to validate the assay for each new one. In general, to generate a TT-comet, deproteinized sperm DNA is initially subjected to a neutral electrophoresis that leads to mobilization of free DNA fragments associated with DSBs. After turning the microgels 90°, an alkaline electrophoresis results in the DNA migration due to both SSB and alkali-labile sites, which extend comet tails. Altogether, the differentiation of levels and types of DNA damage in sperm cells by using the TT-comet generates information to better understand male infertility.

Final remarks

Taken together this review reflects that the comet assay combines toxicological relevance, simplicity, versatility, cost-effectiveness, and high-throughput potential. Its persistent acceptance is based on continuous improvements in efficiency and standardization of existing protocols. The still growing number of publications each year demonstrates the importance and wide range of application of the comet assay. The present review highlighted methodical aspects with special regard to the influence of basic parameters and critical steps as well as the evaluation and scoring of the comets. Also oxidative DNA damage and repair were addressed. An overview of the current work dealing with the comet assay demonstrates that this method is suitable for any type of tissue and cell which makes it possible to examine a wide range of end points and answering important questions from almost any kind of research area. But the evaluation of the current work also indicates that there is still a need to further strengthen the standardization of comet assay protocols to ensure the generation of comparable results. After 30 years of the comet assay, we are looking forward to new challenges, really standardized protocols, and automated comet scoring systems as well as many unexpected new developments and applications. The comet assay will continue to accompany the scientific community interested in DNA damage and repair.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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