30 YEARS OF THE COMET ASSAY: AN OVERVIEW WITH SOME NEW INSIGHTS

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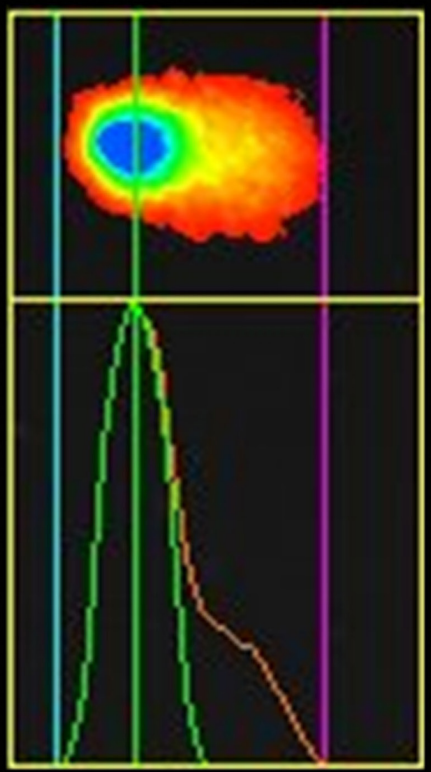

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30 YEARS OF THE COMET ASSAY: AN OVERVIEW WITH SOME NEW INSIGHTS

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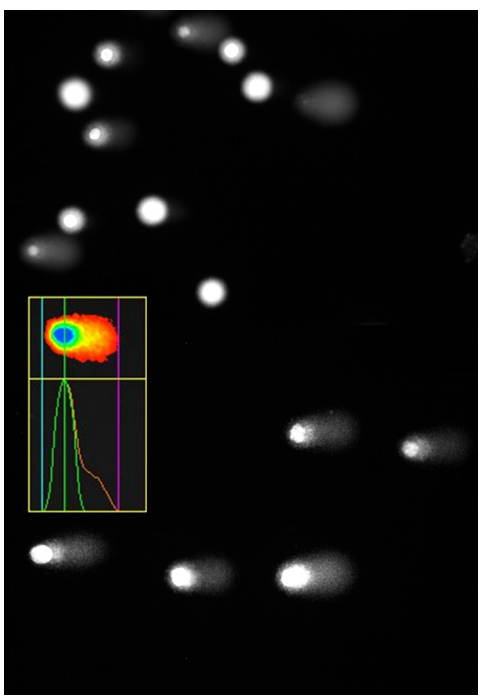

Typical comet images obtained from human lymphocytes treated with hydrogen peroxide; screenshot taken with Perceptive Instruments' Comet Assay IV software.
Image by Sabine Langie.

By means of this 'Frontiers in Genetics' research topic, we are celebrating 30 years of the Comet Assay. The first paper on this single-cell gel electrophoresis assay was published in 1984 by O. Ostling and K.J. Johanson (Biochem. Biophys. Res. Commun. Vol.123: 291-298). The comet assay is a versatile and sensitive method for measuring single- and double-strand breaks in DNA. By including lesion-specific enzymes in the assay, its range and sensitivity are greatly increased, but it is important to bear in mind that their specificity is not absolute. The comet assay (with and without inclusion of lesion-specific enzymes) is widely used as a biomarker assay in human population studies - primarily to measure DNA damage, but increasingly also to assess the capacity of cells for DNA repair. Ostling and Johanson (Biochem. Biophys. Res. Commun., 1984) were also the first to report experiments to measure DNA repair, by simply following the decrease of DNA damage over time after challenging cells with ionising radiation. However, this approach is time-consuming and laborious as it requires an extended period of cell culture and is therefore not ideal for biomonitoring studies, which typically require high-throughput processing of many samples. As an alternative approach, the in vitro comet-based repair assay was developed: a cell extract is incubated with a DNA substrate containing specific lesions, and DNA incisions accumulate. The in vitro comet-based repair assay has been modified and improved over

the past decade: it was first devised to measure base excision repair of oxidised purines in lymphocytes (Collins et al., Mutagenesis, 2001), but has since been adapted for other lesions and thus other repair pathways, as well as being applied to tissue samples in addition to cell suspensions.

Even after 30 years, the comet assay is still in a growth phase, with many new users each year. Many questions are repeatedly raised, which may seem to have self-evident answers, but clearly, it is necessary to reiterate them for the benefit of the new audience, and sometimes being forced to think again about old topics can shed new light. Different applications of the comet assay are discussed in this special issue, including: genotoxicity testing in different organisms, human biomonitoring, DNA repair studies, environmental biomonitoring and clinical studies. Furthermore, we consider and where possible answer questions, including the ones raised by Raymond Tice at the 8th International Comet Assay Workshop in Perugia (Italy 2009): What is the spectrum of DNA damage detected by the various versions of the comet assay?; What are the limitations associated with each application?; What should be done to standardize the assay for biomonitoring studies?; Can the comet assay be used to monitor changes in global methylation status?; What cell types are suitable for detecting genotoxic substances and their effects in vivo and in vitro?; Can the assay be fully automated?; and more. So this 'Frontiers in Genetics' research topic is written for the beginner as well as for the experienced users of the comet assay.

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Table of Contents

New applications of the comet assay

Standardisation of the comet assay: reference standards and normalisation

Data interpretation and statistics

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The comet assay: past, present, and future

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The alkaline comet assay (single cell gel electrophoresis) is the most widely used method for measuring DNA damage in eukaryotic cells (Neri et al., 2015). It detects strand breaks (SBs) and alkali-labile sites at frequencies from a few hundred to several thousand breaks per cell—a biologically useful range, extending from low endogenous damage levels to the extent of damage that can be inflicted experimentally without killing cells. Digestion of the nucleoids, after lysis, with certain lesion-specific repair endonucleases allows measurement of damage other than SBs; notably, formamidopyrimidine DNA glycosylase (FPG) has been widely used to detect altered purines, which are converted to breaks by the enzyme. Recently, (Cortés-Gutiérrez et al., 2014) developed a two-dimensional Two-Tailed comet assay (TT-comet) that can differentiate between single-stranded (SSBs) and double-stranded DNA breaks (DSBs) in the same comets in sperm.

Since the first report by Ostling and Johanson (1984) the comet assay has been widely used in genotoxicity testing of chemicals, in both *in vitro* and *in vivo* models. An advantage with the latter is that cells from various tissues can be studied, in a wide variety of eukaryotic organisms. During the last 15 years, the comet assay has been extensively used in *Drosophila melanogaster* to test the genotoxicity of chemicals (Gaivão and Sierra, 2014). This approach is very useful since *Drosophila melanogaster* is a valuable model for all kinds of processes related to human health, including DNA damage responses.

The use of plants as well as a wide range of terrestrial and aquatic species in the comet assay has dramatically increased in the last decade (Costa et al., 2014; de Lapuente et al., 2015; Santos et al., 2015), particularly in environmental risk assessment (ERA). A recent validation study has indicated that the *in vitro* comet assay combined with FPG may be an effective complementary line-of-evidence in ERA even in particularly challenging natural scenarios such as estuarine environments (Costa et al., 2014).

During the past decade the production and use of nano-sized materials has significantly increased, and as a consequence so has human exposure to these types of materials. Identifying and understanding the hazards of nanomaterials (NMs) in relation to human health is not a simple matter. Not only is the chemical composition of NMs responsible for their genotoxicity, but also shape, specific surface area, size, size distribution, and zeta potential determine the effects of these materials on the genome. Although there is still a debate about the suitability of standard genotoxicity assays for studying the effects of NMs, so far the most used method in nanogenotoxicology, thanks to its robustness, versatility, and reliability, has been the comet assay (Azqueta and Dusinska, 2015). In addition to investigating the genotoxicity of radiation and various chemicals, the plant comet assay has recently also been used to study the genotoxic impact of NPs (Santos et al., 2015).

A further application of the comet assay is as a valuable experimental tool for human biomonitoring as well as in clinical studies. Collecting blood or tissues is not always feasible in all human subjects, and other sources of cells that can be collected non-invasively have been tested

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with the comet assay; for example, various types of epithelial cells (Rojas et al., 2014) as well as sperm (Cortés-Gutiérrez et al., 2014; Brunborg et al., 2015).

In parallel with the development of the comet assay for DNA damage measurement, assays for DNA repair—an essential element in the genotoxic cellular response—have been developed. The simplest approach to DNA repair measurement is to treat cells with a DNA-damaging agent and then to incubate them to allow repair to proceed, measuring the amount of damage remaining at intervals. An alternative, biochemical approach to assessing repair capacity was described in 1994 (Collins et al., 1994), and since then various modified versions of the assay to measure both base excision repair (BER) and nucleotide excision repair (NER) have been published (reviewed by Azqueta et al., 2014). This biochemical approach has been applied to study the effects of environment, nutrition, lifestyle, and occupation on DNA repair capacity, in addition to clinical investigations (Azqueta et al., 2014).

This alternative *in vitro* approach to DNA repair assesses the repair activity of a cell extract on a DNA substrate containing defined lesions. The comet assay is used to follow the accumulation of DNA breaks (repair intermediates) with time of incubation. Recently, Slyskova and colleagues were the first to apply the *in vitro* DNA repair assays for BER and NER successfully on human tissue samples; specifically, colorectal carcinoma biopsies (Slyskova et al., 2012, 2014).

A different kind of DNA repair assay, allowing cells embedded in the gel to repair before lysis, was recently adopted to study DNA repair kinetics in more detail; specifically, to study the regulation of BER proteins by post-transcriptional modifications (Nickson and Parsons, 2014). Yet another way to study DNA repair, at the level of specific genes, is with the comet-FISH technique, which makes use of fluorescent-labeled DNA probes that will hybridize to the single-stranded DNA in the comet tail. McAllister et al. (2014) used this method to study preferential strand break repair in bulk DNA as well as in selected regions with actively transcribed genes.

Studying the kinetics of repair of induced damage will help in our understanding of cellular responses to genotoxic chemicals. Moreover, the significance of DNA repair as a player in the (anti)carcinogenic process can be elucidated by looking at repair at the level of specific cancer target tissues. Regulation of repair—and other aspects of the cellular response to genotoxic compounds—is likely to involve epigenetic mechanisms and the comet assay has been adopted successfully to measure changes in the global DNA methylation pattern in individual cells under various growth conditions (Lewies et al., 2014).

Per cent tail DNA is recommended as the best descriptor for DNA break frequencies, as the comets referred to—and extent of damage—can easily be visualized. However, many researchers still prefer the use of tail moment (Møller et al., 2014). In fact the two descriptors are similarly influenced by assay conditions (Azqueta et al., 2011; Ersson and Möller, 2011).

Variability in the comet assay is an important issue, whether it arises from the use of different protocols, or from uncontrollable or random experimental variation. The inclusion of reference standards in all experiments is recommended, especially when a large number of samples—from a biomonitoring trial, for example—are analyzed on different occasions. Reference standards are cells with a known amount of DNA damage; either untreated cells (negative control), X-ray-exposed cells (positive control), or cells treated with photosensitizer plus light (positive control for assays including FPG-incubation), batch-prepared and frozen as aliquots. If substantial variation occurs in the standards in a run of experiments, sample results can be normalized (Collins et al., 2014). If reference standards are exchanged between laboratories, results from these laboratories can more easily be compared.

Reference standard cells are normally set in gels in parallel to sample gels. Internal standards—i.e., standard cells in the same gel as sample cells—would be ideal; but it is of course essential to be able to distinguish the two types of cell. Fish cells that are either larger or smaller in genome size compared to human cells have successfully been adopted for this purpose (Brunborg et al., 2015). These reference cells can be used in combination with a standard or calibration curve (established with cells given different doses of ionizing radiation), enabling a more precise quantification of DNA lesions expressed as a DNA break frequency rather than % tail DNA.

Statistics are an important tool in all applications of the comet assay, to check whether small differences occur by chance. Concise descriptions of statistical analysis and recommendations for tests have been published (Lovell et al., 1999; Lovell and Omori, 2008). Møller and Loft (2014) remind us that to keep the comet assay statistical analysis simple, appropriate study design and statistical power should be carefully considered when planning experiments.

As with all biological assays, data integration is crucial to interpret the comet assay results within the bigger picture. Integration of information provided by the comet assay with other DNA-damage indicators and cellular responses (e.g., oxidative stress, cell division, or cell death) has been applied both in ERA (Costa et al., 2014; Santos et al., 2015) as well as human (biomonitoring) studies (e.g., Langie et al., 2010; Slyskova et al., 2012). Also including "omics" data will aid in unraveling the mode of action of genotoxic compounds (Slyskova et al., 2012, 2014; Santos et al., 2015)—though it is worth pointing out that several studies have shown that phenotypic measures of DNA repair do not necessarily correlate with genomic or transcriptomic data (Collins et al., 2012; Slyskova et al., 2012, 2014); the different approaches should be regarded as complementary.

Even after three decades of development and modification, the comet assay is still a rather simple, versatile but labor-intensive assay. Various high throughput modifications of the assay were recently reviewed (Brunborg et al., 2014). Both *in vivo* and *in vitro* applications would gain great advantage from further improvements in efficiency, standardization of protocol, and throughput. Automation and miniaturization are common strategies in many areas of biology, allowing orders-of-magnitude changes in the numbers of samples analyzed per experiment, reducing subjective bias, and enhancing reproducibility.

So—what can we hope for in the next 30 years? Acceptance of the *in vitro* comet assay for genotoxicity

testing, inexpensive automated comet scoring to save researchers from interminable microscope viewing, protocol standardization (perhaps) and reliable internal reference standards, more human biomonitoring studies of DNA repair (accepting that phenotypic assays have an important place alongside genomics and transcriptomics), environmental monitoring using a variety of animal and plant species; and many more unpredictable developments and applications.

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OPINION
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The use of the comet assay for the evaluation of the genotoxicity of nanomaterials

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Keywords: comet assay, regulatory toxicity, genotoxicity, nanomaterials, OECD WPMN

Introduction

The accelerating production and use of engineered nanomaterials (NMs) raises questions about the safety of this new technology. To avoid the possible hazards associated with NMs requires proper regulation. How should toxicity testing be addressed and can standard tests for assessment of safety of chemicals be applied to NMs? What are the major limitations of NM safety testing and is the current regulatory testing strategy suitable also for NMs? Can existing tests be fully adapted or should new methods be developed to suit the unique properties of NMs?

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Nanomaterials and Nanoparticles

NMs are nanometer-scale materials that present at least one of their dimensions 100 nm or less. Nanoparticles are NMs with all three dimensions within 100 nm (http://ec.europa.eu/environment/chemicals/nanotech/index.htm#definition; Magdolenova et al., 2014). (In nanomedicine nanoscale particles larger than 100 nm are still considered as NMs.) The small size makes NMs very reactive, as the relative increase in surface area leaves a higher number of molecules to react with the environment. Thus, in their physical, chemical and biological properties, NMs are very different from the bulk material with the same chemical composition. On account of these unique characteristics they have found applications in a wide range of areas: technology, energy, construction, electronics, agriculture, optics, paint, textiles, food, cosmetics, medicine…The production of NMs has impressively increased in the last two decades and nowadays humans are exposed to an unknown amount of a great variety of NMs used in the production of daily life products.

Genotoxicity Assessment of Nanonanomaterials

The same characteristics that make NMs interesting for many applications can also lead to toxicity. Thus concern about the potential harmful effect of NMs on human health has increased. NMs can enter the cell, interact with cell components and persist in cells with consequent chronic toxicity. A new research area that explores the potential toxicity of NMs in human and the environment is nanotoxicology. Within the nanotoxicology field, nanogenotoxicology studies the effect of NMs on DNA.

NMs can also enter into the nucleus, intentionally (i.e., in nanomedicine) or unintentionally, and there might interact with DNA, causing genetic damage (DNA breaks, altered bases or

chromosomal damage). They can also reach the nucleus during mitosis and interfere with the microtubules, causing clastogenic effects. NMs can interact with cellular and mitochondrial membranes or alter mitochondrial function, provoking the production of reactive oxygen and nitrogen species and inducing DNA oxidation. Inflammation produced by NMs in tissues can also affect DNA. NMs can even induce genotoxic effects by depleting the antioxidant defenses or altering the DNA repair systems. All these events may result in pre-mutagenic lesions that can lead to mutations and possibly to cancer and other diseases.

Genotoxicity endpoints are crucial in assessing the safety of chemicals. The Organisation for Economic Co-operation and Development (OECD[1]) has published guidelines for several validated and standardized *in vitro* and *in vivo* methods including genotoxicity assays covering different endpoints (http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm). It is clear that the strategies and the standardized protocols used for characterizing the potential toxicity of chemicals might not be fully suitable for assessing the safety of NMs. NMs can interfere with assay components or detection systems of standard toxicity tests (Guadagnini et al., 2015). In 2006, the OECD created a Working Party on Manufactured Nanomaterials (WPMN) with the aim to review the OECD guidelines for genotoxicity and assess their suitability for NMs.

In 2009 the OECD WPMN published a report recommending the bacterial reverse mutation (Ames test) (OECD TG 471[2]), mammalian chromosome aberration (OECD TG 473[3]) and mammalian cell gene mutation (OECD TG 476[4]) tests for *in vitro* testing; and the mammalian erythrocyte micronucleus (OECD TG 474[5]), mammalian bone marrow chromosome aberration (OECD TG 475[6]) and mammalian liver unscheduled DNA synthesis (UDS) (OECD TG 486[7]) tests for *in vivo* testing (OECD, 2009[8]). However, the Ames test is not suitable for NMs because there is limited or no penetration through the bacterial wall. Thus it is not surprising that NMs exhibiting a positive response in *in vitro* mammalian cell tests have shown negative results in the Ames test (Landsiedel et al., 2009; Doak et al., 2012; Jomini et al., 2012; Woodruff et al., 2012). In the case of the *in vitro* micronucleus test, the interaction between cytochalasin B and NMs is a limiting factor. Cytochalasin B inhibits cytokinesis and is used to generate the binucleated cells but it also inhibits endocytosis, an important mechanism of uptake of NMs into the cell (Doak et al., 2009; Gonzalez et al., 2011). NMs were also seen in the slides when high concentrations of NMs were tested (Pfaller et al., 2010) though this does not seem to be a problem.

Last year, the OECD WPMN published a new report about the genotoxicity evaluation of NMs (OECD, 2014[9]). The Ames test was not recommended for the investigations of the genotoxicity of NMs for the reason explained above. Modification of the *in vitro* micronucleus assay was discussed to ensure the exposure of the cells to the NMs in the absence of cytochalasin B.

Although a lot of effort is being made to develop a testing strategy to assess the genotoxicity of NMs in a reliable way, a consensus on regulatory requirements is still needed. According to the last OECD WPMN report there is a need for an assay that identifies and characterizes the DNA damage induced by secondary mechanisms (e.g., oxidative stress induced by inflammation) (OECD, 2014). Moreover, a complete strategy to assess the genotoxicity of NMs should cover different mechanisms and endpoints including assays to detect strand breaks and altered DNA bases.

The Comet Assay in Genotoxicity Testing

The comet assay is widely used in *in vitro* and *in vivo* genotoxicity testing. It measures DNA strand breaks and alkali-labile sites in virtually any eukaryotic cell including cells isolated from tissues. Its modification with DNA repair enzymes, which convert the specific lesions to breaks, makes the assay more versatile (e.g., formamidopyrimidine DNA glycosylase, FPG, detects 8-oxoguanine and other purine oxidation products). The *in vivo* comet assay, in its standard version, has been validated and the OECD guideline was published last September (OECD TG 489[10]); this assay can be applied in many animal tissues, a great advantage when organ-specific toxicity is expected or investigated. The role of the *in vitro* comet assay in regulatory toxicity is currently not defined but efforts are being made to validate it. Nevertheless, it is recommended as an appropriate test under the Registration, Evaluation, Authorisation and Restriction of Chemicals Substances programme of the European Commission (REACH), is accepted by the European Food Safety Authority (EFSA) and is widely used for screening novel cosmetics and pharmaceuticals. It is also the most used assay in assessing the genotoxic potential of NMs. Magdolenova et al. (2014) reviewed genotoxicity techniques used in 112 papers, published from 2000 to 2012, where the potential genotoxicity of NMs was studied. Similarly, Azqueta et al. (2014) reviewed 102 papers where the genotoxicity of NMs with a potential application in medicine was assessed. According to the results of both reviews (**Table 1**), where the authors of this paper were directly involved, the comet assay and the micronucleus test are

[1]OECD guidelines for the testing of chemicals; http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

[2] OECD. Test Guideline 471. Bacterial reverse mutation test, 1997. In OECD Guidelines for Testing of Chemicals.

[3]OECD. Test Guideline 473. *In vitro* mammalian chromosome aberration test, 2014. In: OECD Guidelines for testing of chemicals.

[4]OECD. Test Guideline 476. *In vitro* mammalian cell gene mutation test, 1997. In: OECD Guidelines for testing of chemicals.

[5]OECD. Test Guideline 474. Mammalian erythrocyte micronucleus, 2014. In: OECD Guidelines for testing of chemicals.

[6]OECD. Test Guideline 475. Mammalian bone marrow chromosome aberration test, 2014. In: OECD Guidelines for testing of chemicals.

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[8]OECD. 2009. Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials. Series of Safety of Manufactured Nanomaterials No. 15.

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[10]OECD. Test Guideline 489. *In vivo* mammalian alkaline comet assay, 2014. In: OECD Guidelines for testing of chemicals.

TABLE 1 | Results obtained by Magdolenova et al. (2014) and Azqueta et al. (2014).

	(Magdolenova et al., 2014) 112 papers	(Azqueta et al., 2014) 102 papers
In vitro studies	94	81
Comet assay	**58**	**52**
Micronuclei assay	**31**	**30**
Chromosome aberration test	**10**	**9**
Ames test	**13**	**9**
γ-H2AX by immunostaining	**–**	**9**
In vivo studies	22	16
Micronuclei assay	**14**	**11**
Comet assay	**9**	**6**
Sporadic techniques	Chromosome aberration assay *in vivo*, gene mutation assay, sister chromatid exchange, γ-H2AX assay and others.	Chromosome aberration assay, gene mutation assay, sister chromatid exchange, γ-H2AX assay by immunostaining *in vivo* and others.

Note that some papers can include results from both in vitro and in vivo studies and also different assays.

the most used techniques *in vitro* and *in vivo*, the comet assay being the most used in *in vitro* and the micronucleus test in *in vivo* studies.

Some interactions of NMs with the comet assay have been described though most of them are hypothetical. Some authors have described the presence of NMs in the comets, which implies that they were also present during the performance of the assay and could have interacted with the naked DNA inducing artificial additional damage (Stone et al., 2009; Karlsson, 2010). However, Magdolenova et al. (2012) found with 5 NMs that their presence in the gel does not affect the comet tail. Karlsson et al. (2015) discussed different possibilities of interference of NMs with the assay and concluded that under normal experimental conditions the additional damage is unlikely to be significant. NMs present in the comets could also interfere with the staining of the comets. Karlsson et al. (2015) suggest that, though there is no indication of this phenomenon, the visual scoring of the comets (rather than computerized image analysis) can be useful.

Interference of FPG with the comet assay (Kain et al., 2012) is also unlikely when applied correctly in the test (Magdolenova et al., 2012). On the other hand, caution is needed with photocatalytic NMs as they can induce additional breaks when the slides are exposed to normal light during their processing (Karlsson et al., 2015).

The comet assay has not been mentioned by the OECD WPMN as a potential appropriate test for testing NMs. The *in vitro* version of the assay does not have an OECD guideline yet though an *in vivo* versions was accepted in September 2014 (OECD TG 489), about 2 months before the publication of the OECD WPMN report on genotoxicity evaluation of MNs (OECD, 2014). The comet assay is considered as an indicator test detecting intermediate DNA lesions that can be repaired or fixed into mutations. Nevertheless, both *in vitro* and *in vivo* comet assays can complete the strategy to assess the genotoxicity of NMs since with the lesion-specific enzymes DNA lesions such as oxidized bases can be detected, additionally to DNA breaks. Moreover, the *in vivo* version is also suitable to detect DNA damage induced by secondary mechanisms such as oxidative stress induced via inflammation in several organs.

Conclusion

Nanotechnology promises enormous benefits to society but also brings new challenges. One of them is the safety of new materials, and consequently there is a growing need for NM toxicity testing, Recent regulations based on hazard assessment of chemicals are not fully fit for purpose for testing NMs as current methods to assess NM toxicity do not always take into account the specific features of NMs. For example, some OECD-recommended tests for genotoxicity (Ames test), are not applicable, or need modification to avoid interference of tested NMs with the test system (micronucleus test). The comet assay has proved to be a sensitive and relatively simple method to study specific DNA lesions such as single and double strand breaks, oxidation and alkylation lesions or cross links. It is so far the most used method in nanogenotoxicology and has great potential to be included in a test battery due to its robustness, versatility and reliability.

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Drosophila comet assay: insights, uses, and future perspectives

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The comet assay, a very useful tool in genotoxicity and DNA repair testing, is being applied to *Drosophila melanogaster* since around 15 years ago, by several research groups. This organism is a valuable model for all kind of processes related to human health, including DNA damage response. The assay has been performed mainly *in vivo* using different larvae cell types (from brain, midgut, hemolymph, and imaginal disk), but also *in vitro* with the S2 cell line. Since its first application, it has been used to analyze the genotoxicity and action mechanisms of different chemicals, demonstrating good sensitivity and proving its usefulness. Moreover, it is the only assay that can be used to analyze DNA repair in somatic cells *in vivo*, comparing the effects of chemicals in different repair strains, and to quantitate repair activities *in vitro*. Additionally, the comet assay in *Drosophila*, *in vivo* and *in vitro*, has been applied to study the influence of protein overexpression on genome integrity and degradation. Although the assay is well established, it could benefit from some research to determine optimal experimental design to standardize it, and then to allow comparisons among laboratories independently of the chosen cell type.

Keywords: *Drosophila*, comet assay, neuroblast cells, hemocytes, midgut cells, genotoxicity, DNA repair

INTRODUCTION

The single cell gel electrophoresis test, or comet assay, was originally developed by Östling and Johanson (1984) as a microelectrophoretic technique to visualize DNA damage in single cells. Subsequently it was improved by Singh et al. (1988), and since then so extensively used that some working-groups were created to standardize its application to mammal and human cells studies (Burlinson et al., 2007; Karlsson, 2010; Azqueta and Collins, 2013; Ersson et al., 2013; Godschalk et al., 2013; Collins et al., 2014).

Its usefulness and easy performance lead to its rapid application to several fields, like genotoxicity analyses (Speit and Hartmann, 1999; Tice et al., 2000; Hartmann et al., 2003; Collins, 2004), human population biomonitoring (Collins et al., 1998; Somorovská et al., 1999; Kassie et al., 2000; Møller et al., 2000; Faust et al., 2004; Hoffmann et al., 2005; Burlinson et al., 2007; Dusinska and Collins, 2008; Uriol et al., 2013) and DNA repair (Collins and Horváthová, 2001; Collins et al., 2001; Collins and Gaivão, 2007; Gaivão et al., 2009; Dusinska and Collins, 2010). Because of this, it was also applied to other organisms, using different cell types (Menke et al., 2001; Dixon et al., 2002; Lee and Steinert, 2003; Jha, 2008; Dhawan et al., 2009; Ventura et al., 2013).

Surprisingly, its application to *Drosophila melanogaster* was rather late, despite the fact that this organism is one of the most valuable higher eukaryotic model organism, for all kind of processes and situations related to human health (Reiter et al., 2001; Koh et al., 2006; Wolf et al., 2006; Khurana et al., 2006; Rand, 2010), including the *in vivo* DNA damage response processes (Søndergaard, 1993; Vogel et al., 1999; Sekelsky et al., 2000; Vecchio, 2014).

The first attempt to apply the comet assay to *Drosophila in vivo* was performed by Gaivão (1999) in her Ph.D. Thesis, checking the availability of imaginal disk and brain ganglia cells. In the first published work, appeared 3 years later, the comet assay was performed with brain ganglia cells from third instar larvae (Bilbao et al., 2002). As with other organisms, several cell types, apart from the brain cells, have been used to carry out this assay in *Drosophila in vivo*, such as midgut cells (Mukhopadhyay et al., 2004; Siddique et al., 2005a; Sharma et al., 2011), hemocytes (Carmona et al., 2011a), and imaginal disk cells (Verma et al., 2012).

Most of these authors used the comet assay for its original purpose, the *in vivo* analyses of genotoxicity and DNA repair. But more recently, this assay has also been used to study genotoxicity *in vitro* (Guanggang et al., 2013), to analyze the influence of protein overexpression on genome integrity *in vivo* (Plyusnina et al., 2011; Brennan et al., 2012; Verma et al., 2012) and *in vitro* (Radyuk et al., 2006), and very recently to quantitate DNA repair activity *in vitro* (Rodríguez et al., submitted).

In this mini-review we aim to present available information about the comet assay in *Drosophila*; outlining the type of cells and insights into its technical performance, its uses *in vivo* and *in vitro*, and its spread availability as a useful tool and future perspectives.

INSIGHTS

BRAIN CELLS

The *Drosophila* comet assay using brain ganglia cells was developed at the University of Oviedo (Spain) by Isabel Gaivão and the group of L. María Sierra and M. A. Comendador (Gaivão, 1999;

Bilbao et al., 2002). Our aim was to develop a tool to study both genotoxicity and *in vivo* DNA repair in somatic cells.

The developed protocol included the use of third instar larvae (developed 24 h at 24°C and five additional days at 21°C) treated in the food during 12 h. Brain ganglia were extracted, and cells were mechanically individualized, shredding the tissue with tungsten wires, and suspended in Ringer's buffer (Bilbao et al., 2002; García-Sar et al., 2008, 2012; Rodríguez et al., submitted). Cells were embedded in 0.5% low melting point agarose (LMPA), three agarose layers were prepared, and cells were disrupted during 2 h with a lysis solution containing *N*-lauroylsarcosine sodium salt (*N*-LS), 0.77%, and dimethyl sulfoxide (DMSO) 10%. Denaturation was performed at pH 12.6, for 20 min, and electrophoresis was set at 0.9 V/cm, for 20 min. After neutralization and fixation, slides were stained with ethidium bromide (0.4 μg/mL), with Vectashield® fluorescence protector (Vector Laboratories, Inc., Burlingame, CA, USA) to avoid fluorescence decay (**Table 1**). A very detailed protocol was recently published (Sierra et al., 2014).

Microscope photos were analyzed with the Komet 5 software program (Kinetic, England), collecting data on % tail DNA, tail length, and tail moment, although the analyses were carried out with the tail moment parameter because it increased linearly with the amount of DNA damage and was the best to detect statistically significant differences. The wild-type OregonK *Drosophila* strain was used as a standard, since it is rather sensitive to the action of DNA damaging agents in somatic cells (Gaivão and Comendador, 1996). Under all these conditions, the comet assay yielded spontaneous DNA damage measurements of 6.5 ± 0.5 for tail moment and of 30 ± 1.25 for % tail DNA.

Recently, we have developed a technical variation of this protocol to be able to quantitate DNA repair activities *in vitro*. This variant consists on the incubation of nucleoid DNA with cell-free protein extracts from repair-efficient and deficient-strains, after the lysis step (Rodríguez et al., submitted).

Plyusnina et al. (2011) also used brain cells to perform the comet assay. They disaggregated them mechanically in Poels' salt solution (PSS). Cells were embedded in 0.75% LMPA, lysis was performed for 1 h, with a buffer without *N*-LS or DMSO. Denaturation was carried out at pH 13 for 10 min, followed by electrophoresis at 15 V–300 mA for 10 min. Nuclei were stained with acridine orange. Comet images were analyzed with the Comet Score™ software (TriTek Corporation, USA), and the parameter for analysis was the Olive tail moment. The wild-type strain was Canton-S and the values of spontaneous DNA damage measurements were approximately 1.2 units of the analyzed parameter.

HEMOCYTES

The comet assay using hemocytes from *Drosophila* was developed by the group of R. Marcos at the Autonomous University of Barcelona (Spain). In this protocol, 72 ± 2 h old larvae (developed at 24°C) were treated for 24 h. Since hemocytes are individual cells, they were just collected in phosphate buffered saline (PBS), with 0.07% phenylthiourea (Carmona et al., 2011a,b,c; Sabella et al., 2011).

Cells were embedded in 0.75% LMPA, and two agarose layers were prepared. Lysis buffer contained *N*-LS 1% (Carmona et al., 2011a,b,c), or DMSO 10% (Sabella et al., 2011). Lysis time was 2 h. Small variations on the denaturation time and the electrophoresis conditions were performed (**Table 1**). Nucleoids were stained with DAPI (1 μg/mL). Detailed protocols for this assay are available (Marcos and Carmona, 2013; Sierra et al., 2014).

Comets were analyzed with the Komet 5 software program, and results were mostly expressed as % tail DNA (Carmona et al., 2011a,b,c), although DNA damage was also measured as percentage of damaged nuclei (Sabella et al., 2011). The standard wild-type strain used was OregonR, an insecticide resistant strain with high levels of cytochrome P450 and xenobiotic metabolism (Hällström et al., 1984). With this protocol, the highest % tail DNA detected for spontaneous DNA damage was 18.93 ± 0.84 (Carmona et al., 2011c).

MIDGUT CELLS

The comet assay with midgut cells was developed by the group of A. Dhawan and D. K. Chowdhuri at the CSIR-Indian Institute of Toxicology Research, formerly Industrial Toxicology Research Center (India). They also developed the enzymatic brain cell disaggregation protocol. Mid-gut tissue, with or without brain ganglia, from third instar larvae treated for different times were explanted in PSS buffer. Cells were enzymatically individualized, incubating 15 min with collagenase (0.5 mg/mL) in PBS. Treatment times varied from 12 to 74 h (**Table 1**; Mukhopadhyay et al., 2004; Siddique et al., 2005a,b, 2008, 2013; Mishra et al., 2011, 2013, 2014; Sharma et al., 2011, 2012; Shukla et al., 2011).

Cells were embedded in 0.75% LMPA, with two or three agarose layers. Lysis buffer did not contained *N*-LS, or DMSO, and lysis time was 2 h. As presented in **Table 1**, the denaturation step was mainly performed at pH > 13 during 10 min, although in two works this step was performed at neutral conditions, pH 8.5 for 60 min (Sharma et al., 2011; Mishra et al., 2013). In these two cases electrophoresis was also set up differently from the more standard 0.7 V/cm during 15 min (**Table 1**). Staining was carried out with ethidium bromide (20 μg/mL), for 10 min.

Some of the works carried out at the CSIR-Indian Institute of Toxicology Research analyzed three comet parameters, % tail DNA, tail length, and Olive tail moment (Mukhopadhyay et al., 2004; Siddique et al., 2005a,b), and in others only the % tail DNA was used for result analyses. The Komet 5 software program was throughout used for photo analysis, except by Siddique et al. (2013), who used the Comet Score™ software, v1.5, to analyze tail length. The standard wild-type strain was OregonR. With this protocol, % tail DNA varied from 6 to 10%, with errors lower than 1%, and Olive tail moment varied from 0.7 to 1.5, with errors under 0.12.

IMAGINAL DISK CELLS

Imaginal disk cells have also been used to carry out the comet assay *in vivo* in *Drosophila* (Verma et al., 2012). In this case, cell disaggregation was performed enzymatically, as described earlier for midgut cells (see Midgut Cells).

Table 1 | Methodological details, analyzed agents, and results of genotoxicity and/or DNA repair studies carried out in *D. melanogaster* with the comet assay.

Cell type	Treat. time (h)	% Agarose	Denaturat pH/time (min)	Electrophoresis V/cm/time (min)	Staining	Strain	Agents	Results	Reference
Brain cells	12	0.5	12.6/20	0.9/20	EthBr 40 μL (0.4 μg/mL)	*OregonK, mus201*	cDDP	+	García-Sar et al. (2008, 2012)
						OregonK, mus201, mus308,double mut	MMS	+	Rodríguez et al. (submitted)
			10–12.6/20			*OregonK, mus201, mus308*	MMS	+	Bilbao et al. (2002)
							EMS	+	
							ENU	+	
Hemocytes	24 ± 2	0.75	13/25	0.7/20	DAPI 20 μL (1 μg/mL)	*OregonR*	EMS	+	Carmona et al. (2011a)
							Cr(VI)-$K_2Cr_2O_7$	+	
							γ-rays	+	
							$PbCl_2$	−	Carmona et al. (2011b)
							$Pb(NO_3)_2$	+	
							$NiCl_2$	−	Carmona et al. (2011c)
							$NiSO_4$	+	
			13/20	0.73/25			AuNP	+	Sabella et al. (2011)
Mid-gut cells	74	0.75	> 13/10	0.7/15	EthBr 75 μL (20 μg/mL)/ 10 min	*OregonR*	Cypermethrin	+	Mukhopadhyay et al. (2004)
							Industrial waste leachates	+	Siddique et al. (2005b)
	24					*OregonR*	EMS	+	Siddique et al. (2005a)
							MMS	+	
							ENU	+	
							CP	+	
						OregonR plus FPG and EndoIII enzimes	H_2O_2	+	Shukla et al. (2011)
							$CdCl_2$	+	
							$CuSO_4$	+	
	24/48					Transgenic (*hsp70-lacZ)Bg*	Graphene-Cu_2O nanocomposite	+	Siddique et al. (2013)

(Continued)

Table 1 | Continued

Cell type	Treat. time (h)	% Agarose	Denaturat pH/time (min)	Electrophoresis V/cm/time (min)	Staining	Strain	Agents	Results	Reference
	48					*OregonR, mei9, mus201, mus210, mei41, mus207, mus209, mus309*	Cr(III)-$CrCl_3$	−	Mishra et al. (2011)
							Cr(VI)-$K_2Cr_2O_7$	+	
							Dichlorvos	+	Mishra et al. (2014)
	12–48					*OregonR* transgenic *hsp70, hsp83, hsp26* plus FPG and EndoIII enzimes	Endosulfan	+	Sharma et al. (2012)
	48/72					*OregonR, mei41, mus201, mus308, rad54*	Industrial waste leachates	+	Siddique et al. (2008)
	48		8.5/60	14V-100mA/60		*OregonR*	CP	+	Sharma et al. (2011)
							BLM	+	
							cDDP	+	
							Cr(VI)	+	
	48/24		8.5/60	14V-60mA/60		*OregonR, ligiV, ku80, spn-A, okr, mre11*	Cr(VI)-$K_2Cr_2O_7$	+	Mishra et al. (2013)
Imaginal disk	48	0.75			Propidium iodide (1 μg/mL)	*OregonR, Act-GAL4/CyO;+/+, Act-GAL4/PolκRNAi;+/+,+/+;pl10R/pl10R*	BLM	+	Verma et al. (2012)
Cultured cells	6/24	0.5	Alkal/30	1/10	SYBR green	*S2* cell line: standard and transfected	Paraquat	+	Radyuk et al. (2006)
							S-nitroso-*N*-acetyl penicillamine	+	
	24/48	1	13/10	1/10	EthBr 40 μL (20 μg/mL)	*S2* cell line	Methomil	+	Guanggang et al. (2013)

The conditions of the comet assay were also those described above (see Midgut Cells) with two exceptions: the lysis buffer contained DMSO 10%, and nuclei were stained with propidium iodide (1 μg/mL). Photos were analyzed with the Comet Score™ software, and DNA damage was quantified using the % tail DNA parameter. The wild-type strain used was OregonR, and the spontaneous values of % tail DNA were around 7 (only a graph was presented).

OTHER CELLS

Spermatocytes were other cell type chosen to perform the comet assay *in vivo*, in this case from *D. simulans* (Brennan et al., 2012). Testes were dissected in PBS. However, with respect to the comet assay, the only information available from this work is that they have used the OxiSelect Comet Assay Kit (from Cell BioLabs, San Diego, CA, USA) to perform it, the Comet Score™ software for image analysis, and a classification of % tail DNA in five categories for the analysis of results.

The comet assay in *Drosophila* was also performed *in vitro* using S2 cultured cells (Radyuk et al., 2006; Guanggang et al., 2013). Cells were treated for 24 h, embedded in 0.5% LMPA, lysed for 30 min, denatured in alkaline conditions for 30 min, electrophoresed at 1 V/cm for 10 min, and stained with SYBR green dye; and the DNA damage was measured classifying the damaged cells in four categories (Radyuk et al., 2006).

Alternatively, cells were treated for 24 or 48 h and embedded in 1% LMPA. Lysis buffer contained DMSO 10%, and lysis time was 30 min. Denaturation at pH 13 for 10 min was followed by electrophoresis 1 V/cm for 10 min. Nucleoids were stained with ethidium bromide (20 μg/mL), and comet photos were analyzed with CASP image analysis system, measuring % tail DNA and tail moment. The values of these parameters for spontaneous DNA damage were 11.57 ± 5.84 for % tail DNA and 2.20 ± 1.24 for tail moment (Guanggang et al., 2013).

USES

GENOTOXICITY AND DNA REPAIR ANALYSIS

It is possible to study DNA repair *in vivo* in *Drosophila* germ cells, male and female ones, since many years ago (Vogel et al., 1996; Hernando et al., 2004). However, it was not possible to study it in somatic cells, with the available *in vivo* SMART assays (Vogel and Nivard, 2001). Because of this, our main aim when designing the first comet assay protocol in *Drosophila* was to develop a tool to study DNA repair *in vivo* in somatic cells (Gaivão, 1999; Bilbao et al., 2002). Consequently, many (but not all) of the works carried out with this assay in *Drosophila* were aimed to study genotoxicity and/or DNA repair in somatic cells *in vivo*.

In addition to its use in the assay design, using model genotoxic agents, and efficient and deficient repair strains (Bilbao et al., 2002), brain cells, obtained with the University of Oviedo protocol, were used to demonstrate the relationship between cisplatin induced adducts and DNA strand breaks (García-Sar et al., 2008), and the influence of the nucleotide excision repair system in this relationship, with the *in vivo* comet repair assay (García-Sar et al., 2012). Very recently, brain cells have been used to implement the *in vitro* comet repair assay in *Drosophila*, to be able to quantitate DNA repair activities *in vitro* (Gaivão et al., 2014), and it was used to check the repair activity of cell free protein extracts obtained from wild-type and repair mutant strains in the repair of methyl methanesulfonate induced DNA damage (Rodríguez et al., submitted).

After checking their use with known inducers of DNA strand breaks (Carmona et al., 2011a), hemocytes were used to demonstrate that not all the salts of lead and nickel were genotoxic (Carmona et al., 2011b,c), but that gold nanoparticles were so (Sabella et al., 2011).

Midgut cells, with or without brain cells, have been used to study oxidative DNA damage, using incubations with FPG and Endo III enzymes (Shukla et al., 2011; Sharma et al., 2012), and to demonstrate the genotoxicity of chromium salts (Mishra et al., 2011, 2013; Sharma et al., 2011), pesticides like cypermethrin (Mukhopadhyay et al., 2004), endosulfan (Sharma et al., 2012), and dichlorvos (Mishra et al., 2014), contaminants as industrial waste leachates (Siddique et al., 2005b, 2008), and nanomaterials like graphene copper nanocomposite (Siddique et al., 2013). In addition, some of these genotoxic agents, like chromium salts, dichlorvos, and industrial waste leachates, were analyzed in different repair conditions, with the *in vivo* comet repair assay (Siddique et al., 2008; Mishra et al., 2011, 2013, 2014), checking the influence of pre- and post-replication DNA repair pathways on their genotoxicity. Other genotoxic agents, like endosulfan and graphene copper nanocomposite, were analyzed in transgenic strains for genes encoding heat shock proteins (hsp), to check responses to xenobiotic stress, and influence of xenobiotic metabolism (Sharma et al., 2012; Siddique et al., 2013).

Analysis of genotoxicity, specifically that of the insecticide methomil, was also the aim of the comet assay performed *in vitro* with S2 culture cells (Guanggang et al., 2013).

OTHER USES

In addition to these studies of genotoxicity and DNA repair, the comet assay *in vivo* in *Drosophila* had been used to study: (i) the influence of GADD45 protein over-expression on longevity and spontaneous DNA damage, as an indication of increased DNA repair activity (Plyusnina et al., 2011); (ii) chromatin integrity in DNA polε mutants exposed to bleomycin (Verma et al., 2012); and (iii) oxidative DNA damage in spermatocytes of *Wolbachia*-infected *D. simulans* flies (Brennan et al., 2012).

Furthermore, the comet assay *in vitro* was used to check the effect of mitochondria ectopic over-expression of dOgg1 and RpS3 proteins on DNA degradation after oxidative damage induction (Radyuk et al., 2006).

FUTURE PERSPECTIVES

Considering the relevance of *D. melanogaster* as an established insect model for human diseases and toxicological research, recommended by the European Centre for the Validation of Alternative Methods (ECVAM), all the results of *in vivo* genotoxicity studies with this organism should be considered as relevant for human health. In this aspect, the comet assay performed *in vivo* is even more important because, in addition to its high sensitivity, it is the only assay that allows the analysis of DNA repair in somatic cells. And, at least theoretically, the comet

assay results should be more easily and directly compared among species.

There is however a possible problem: there are several groups using different protocols, what make comparisons even among *Drosophila* laboratories impossible. So, it is necessary to standardize the basic comet assay protocol. Azqueta et al. (2011) demonstrated in human cells how small changes in some variables, such as agarose concentration, alkaline unwinding time, or electrophoresis conditions, might significantly affect the results. And these are specifically some of the variables that differ between the protocol for brain cells and the rest: LMPA percentage (0.5 vs. 0.75%), lysis buffer composition (*N*-LS and DMSO vs. only *N*-LS or none of them), or denaturation and electrophoresis conditions (more V/cm, compared to the protocol for hemocytes, and more denaturation time and V/cm, compared to the protocol for midgut cells). These differences might explain the higher values of the comet parameters, for spontaneous DNA damage, found with the brain cell protocol, compared to the others, because although some differences might be attributed to the wild-type strain analyzed (OregonK is more sensitive than OregonR), at least in the case of human cells differences due to individuals or cell types were not so relevant (Azqueta et al., 2011). It is then necessary to study the effects of these differences and whether a higher sensitivity is an advantage or a disadvantage.

To help with the required standardization, some of the protocol optimizations performed for other cells and organisms can be tested and applied to *Drosophila*, including its simplification (number of layers, size of gels, or solution compositions) and the high throughput versions, recently developed based on the use of 12 mini-gels on one slide (Shaposhnikov et al., 2010). Additionally, the modified comet assay performed incubating with repair lesion-specific enzymes, as used by Shukla et al. (2011) and Sharma et al. (2012) for oxidative damage, can be extended to other types of damages and repair systems (Collins et al., 2008). This standardization would also clearly help the use of this assay in other types of studies, different from genotoxicity and DNA repair testing.

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The Comet Assay and its applications in the field of ecotoxicology: a mature tool that continues to expand its perspectives

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Since Singh and colleagues, in 1988, launched to the scientific community the alkaline Single Cell Gel Electrophoresis (SCGE) protocol, or Comet Assay, its uses and applications has been increasing. The thematic areas of its current employment in the evaluation of genetic toxicity are vast, either *in vitro* or *in vivo*, both in the laboratory and in the environment, terrestrial or aquatic. It has been applied to a wide range of experimental models: bacteria, fungi, cells culture, arthropods, fishes, amphibians, reptiles, mammals, and humans. This document is intended to be a comprehensive review of what has been published to date on the field of ecotoxicology, aiming at the following main aspects: (i) to show the most relevant experimental models used as bioindicators both in the laboratory and in the field. Fishes are clearly the most adopted group, reflecting their popularity as bioindicator models, as well as a primary concern over the aquatic environment health. Amphibians are among the most sensitive organisms to environmental changes, mainly due to an early aquatic-dependent development stage and a highly permeable skin. Moreover, in the terrestrial approach, earthworms, plants or mammalians are excellent organisms to be used as experimental models for genotoxic evaluation of pollutants, complex mix of pollutants and chemicals, in both laboratory and natural environment. (ii) To review the development and modifications of the protocols used and the cell types (or tissues) used. The most recent developments concern the adoption of the enzyme linked assay (digestion with lesion-specific repair endonucleases) and prediction of the ability to repair of oxidative DNA damage, which is becoming a widespread approach, albeit challenging. For practical/technical reasons, blood is the most common choice but tissues/cells like gills, sperm cells, early larval stages, coelomocytes, liver or kidney have been also used. (iii) To highlight correlations with other biomarkers. (iv) To build a constructive criticism and summarize the needs for protocol improvements for future test applications within the field of ecotoxicology. The Comet Assay is still developing and its potential is yet underexploited in experimental models, mesocosmos or natural ecosystems.

Keywords: Comet Assay, ecotoxicology, piscine model, amphibians, earthworms, mollusks, plants

Introduction

The extraordinary growth in the chemical industry during the second half of the twentieth century has led to the appearance in nature of thousands of new products every year, a large percentage of which have significant biological effects. The presence in the environment of xenobiotics that are biologically active and difficult to break down represents a degree of stress that is frequently unacceptable for living organisms and that is also expressed at the ecosystem level. Both direct and indirect toxic activity can, in certain circumstances, be an important risk factor for the human population as well.

The usual way to approach ecotoxicity testing, according to relevant EPA and OECD guidelines for the testing of chemicals (for example, in the context of REACH normative) or of veterinary drugs, is the use of well-defined tests, in which an array of selected species, representing the main trophic levels, are exposed to a single pollutant under controlled laboratory conditions. Such a standardized approach is necessary to acquire information in a relatively short time, to gather data easy to compare and to interpret and, of course, for regulatory purposes. However, extrapolation to real world is challenging if at all feasible.

Models to study environmental toxicity are a necessary compromise between the control of experimental parameters (through the use of lab-reared substitute species and the setting of a thoroughly controlled exposure scenario) and realism (field or semi-field studies). An entirely different approach is based on the use of native species, which essentially considers pollution as a complex situation and therefore implies a more holistic interpretation of the real conditions of exposure in the field. This kind of study includes the capture of animals and/or the collection of plants, water or soil samples on the field. This approach allows considering interactions among pollutants and also homeostasis. Life-term exposure occurs in a natural context, allowing the action of such modulating factors as discontinuous pattern of pollution, reduction of the animal activity or sheltering. Interpretation of the results, on the other hand, may be particularly difficult in face of the many constraints and confounding factors of the natural environment (Borràs and Nadal, 2004).

The term *mutagen* refers to a substance that induces transmissible changes in DNA structure (Maurici et al., 2005), involving a single gene or a group of genes. Genotoxins are a broader category of substances which induce changes to the structure or number of genes via chemical interaction with DNA and/or non-DNA targets (Maurici et al., 2005). The term *genotoxicity* is generally used unless a specific assay for mutations is being discussed. A large number of assay systems have been established for the measurement of genetic toxicity of chemical and physical agents. The Comet Assay, or Single Cell Gel Electrophoresis (SCGE), is a standard method for determining *in vivo*/*in vitro* genotoxicity. It offers a simple way of evaluating the damage caused by a clastogenic agent by measuring breaks in the DNA chain of animal and plant cells. One of the most striking features of the Comet Assay is the versatility, which allows its application to a wide array of different cell types and matrices. This characteristic, as well as its sensitivity, makes it especially well-suited for ecotoxicological studies, both in the terrestrial and the aquatic compartment.

Although, for different reasons, water has been a privileged scenario for the pioneering studies on environmental genotoxicity, soil remains the primary way of entry into the environment for a number of pollutants, going from agricultural pesticides to veterinary drugs. As a consequence, testing species representative of the trophic chain in both compartments is relevant and necessary to thoroughly assess the genotoxic effects of environmental pollutants. In either case, it is clear that in the last decades the Comet Assay has been applied to a wide range of scenarios, species and ecogenotoxicity assessment approaches. As such, the present paper primarily aims to critically reviewing the application and technical developments of this versatile protocol in the context of ecotoxicology.

Experimental Models

Amphibians

Amphibians are among the most sensitive organisms to environmental changes, mainly due to an early aquatic-dependent development stage and a highly permeable skin. As such, they have been proposed as bioindicators of environmental contamination (Gonzalez-Mille et al., 2013). Environmental contaminants are pointed out as the primary cause in the decline of amphibian populations, hence the importance of evaluating exposure and sublethal effects in amphibian monitoring programs (Gonzalez-Mille et al., 2013). Nonetheless, the application of the Comet Assay in ecotoxicological studies involving these organisms is relatively new. The first work reported dates from 1996 (Ralph et al., 1996). Since then, a number of studies have been conducted that apply the Comet Assay to amphibian cells in adult and larval stages of several species, mainly *Lithobates clamitans* and *Xenopus laevis.* These studies focused mainly on the determination of the exposure effects to several contaminants, such as, for instance: herbicides (Clements et al., 1997; Liu et al., 2006, 2011; Yin et al., 2008; Meza-Joya et al., 2013), pesticides (Feng et al., 2004; Yin et al., 2009; Ismail et al., 2014) and other xenobiotics as methyl methanesulfonate (Ralph et al., 1996; Ralph and Petras, 1998b; Mouchet et al., 2005a). Reports on the effects of the exposure to fungicides (Mouchet et al., 2006a), metals (Wang and Jia, 2009; Zhang et al., 2012), petrochemical contaminants (Huang et al., 2007), Persistent Organic Pollutants (POPs) (Gonzalez-Mille et al., 2013), ethyl methanesulfonate (Mouchet et al., 2005a); benzo(a)pyrene (Mouchet et al., 2005a), sulfur dyes (Rajaguru et al., 2001), antibiotics (Banner et al., 2007; Valencia et al., 2011), and dimethyl sulfoxide (DMSO) (Valencia et al., 2011) may also be found. Additionally, the biomonitoring of contaminated sites recurring to the Comet Assay in amphibians has also been performed, namely, of chemically-polluted lakes (Erismis et al., 2013), coal mines (Zocche et al., 2013), waste dumping sites (Maselli et al., 2010), dredged sediments (Mouchet et al., 2005b), polluted water bodies (Ralph and Petras, 1997, 1998a) and residues from municipal solid waste incineration

(Mouchet et al., 2006b). Studies have also been reported where on sperm cells (Shishova et al., 2013) and the effects of exposure to electromagnetic fields (Chemeris et al., 2004) were assessed by the Comet Assay. Generally, studies are conducted *in vivo* and erythrocytes are the cell type most commonly used.

Piscine Models

Historically, fishes are closely linked with the transposition of the Comet Assay to the field of environmental toxicology, since they are among the first animal models to which the technique was adopted as a biomonitoring tool to assess the genotoxicity of contaminants on wildlife. A pioneering application was carried out by Pandrangi et al. (1995). This study examined the effects of toxic wastes accumulated in the sediment of the Great Lakes (Canada) and the sentinel species selected were the brown bullhead (*Ameiurus nebulosus*) and the common carp (*Cyprinus carpio*). The alkaline procedure developed and reported by Singh et al. (1988) was successfully adapted to fish erythrocytes, albeit the introduction of a few modifications. The authors concluded that the assay "is extremely sensitive and should be useful in detecting DNA damage caused by environmental contaminants." Since 1995, this premonitory statement has been recurrent and increasingly reinforced by an array of scientific publications, exploring a wide diversity of approaches, viz. *in vitro* (Kienzler et al., 2012), *ex vivo* (Santos et al., 2013), *in vivo* (Palanikumar et al., 2013), and *in situ* (Srut et al., 2010) exposures, as well as surveying wild native specimens (Laroche et al., 2013).

To date, more than 300 articles have been published addressing DNA integrity in fish cells through the Comet Assay, making fish by far the most adopted animal group in the framework of environment health assessment. Furthermore, in recent years we have witnessed to an even greater profusion of publications. In 2013, for instance, 43 scientific articles were published (according to a literature search on PubMed) evaluating DNA damage by Comet Assay in piscine models (including fish cell lines) exposed to various potentially genotoxic agents. This vast utilization of fish should also be regarded as reflecting a primary concern of genetic ecotoxicologists over the health status of aquatic ecosystems. As a further evidence of the Comet Assay popularity as a tool for detecting DNA strand breaks in fish (along with other aquatic animals) it should be underlined that this subject has been periodically reviewed in 1998 (Mitchelmore and Chipman, 1998), 2003 (Lee and Steinert, 2003), and 2009 (Frenzilli et al., 2009).

It is well-established that Comet Assay is applicable, virtually, to all species. A clear demonstration of this polyvalence is the finding that, since 1995, this assay was successfully adapted to more than 90 fish species. This wide range of species includes mostly bony fish (Class Osteichthyes), both ray-finned fishes (Subclass Actinopterygii), the overwhelming majority of cases, and lobe-finned fishes (Subclass Sarcopterygii) like *Arapaima gigas* (Groff et al., 2010). The jawless fish (Class Agnatha) are represented with an interesting study with sea lamprey (*Petromyzon marinus*) describing the relationship between sperm DNA damage and fertilizing ability (Ciereszko et al., 2005), while cartilaginous fish (Class Chondrichthyes) are completely unexplored. Bearing in mind that the Comet protocol requires very small cell samples, the technique showed to be suitable for a broad variety of fish sizes, from very small fish (e.g., the mosquitofish *Gambusia holbrooki*; Ternjej et al., 2010), and even fingerlings (e.g., milkfish *Chanos chanos*; Palanikumar et al., 2013), up to bigger species like conger (*Conger conger*; Della Torre et al., 2010).

In what concerns to the type of agent/contaminant tested, the application of Comet Assay in the field of aquatic genotoxicology has accompanied the evolution of other subareas of environmental toxicology involving piscine models. Hence, besides the contaminants traditionally evaluated like POPs (González-Mille et al., 2010), metals (Velma and Tchounwou, 2013), or pesticides (Guilherme et al., 2010), genotoxicologists have shown to be aware to emergent genotoxicants such as pharmaceutical substances (Rocco et al., 2010), endocrine disruptors (e.g., tetrabrombisphenol A; Linhartova et al., 2014), nanoparticles (Taju et al., 2014), biotoxins (Silva de Assis et al., 2013), radionuclides (Stiazhkina et al., 2012), or ultraviolet (UV) radiation (Mekkawy et al., 2010).

Bivalves and Other Molluscs

In recent years, the application of the Comet Assay in molluscs has been springing up. These organisms have long been regarded as prime subjects in biomonitoring programmes worldwide, especially, albeit not exclusively, in aquatic ecosystems. Bivalves, in particular, receive special attention both as sentinel and toxicity-testing subjects and a large array of literature has been published in the last few years. Among these, mussels (*Mytilus* spp.) have become one the most important targets when researching on marine genotoxicants using the Comet Assay (in large part owing to their worldwide distribution and known sensitivity to pollutants), from substance testing to the monitoring of sediments and waters *in situ* and *ex situ* and even recovery assessment following oil spills (Thomas et al., 2007; Almeida et al., 2011; Fernández-Tajes et al., 2011; Pereira et al., 2011; Martins et al., 2012, 2013; Dallas et al., 2013). Research on the genotoxic effects of emerging pollutants, including nanomaterials is also arising (Gomes et al., 2013). Other bivalves, of more local relevance, have been shown to be good candidates, such as the clam *Ruditapes decussatus* in SW Europe (Martins et al., 2013) and the cockle *Cerastoderma edule* (Pereira et al., 2011). In freshwater environments, the green-lipped mussel (*Perna* spp.), the zebra mussel *Dreissena polymorpha* and the Asian clam *Corbicula fluminea* are the most common bivalves in genotoxicity assessment through the Comet Assay (Michel and Vincent-Hubert, 2012; Parolini and Binelli, 2012; Chandurvelan et al., 2013; Michel et al., 2013; dos Santos and Martinez, 2014). Gastropods take the place of bivalves in terrestrial environments and the use of snails (like *Helix* spp.) as effective sentinels for genotoxicants has been demonstrated *in situ* (Angeletti et al., 2013).

Terrestrial Organisms

The fate and effects of pollutants on living organisms may differ in the two compartments. Soils are complex associations with high binding capacity to both inorganic and organic molecules, which may, as well as certain modifications along time

(e.g., aging and weathering), modulate the biological effects of contamination. For these reasons, toxicity to terrestrial species cannot be directly extrapolated from aquatic species, meaning that specific approaches and models are needed to assess the impact of soil pollutants on terrestrial biota (Vasseur and Bonnard, 2014).

The role that filtering organisms, like mussels, play in water is covered in soil by earthworms, which, in addition, are able to move around and prospect its surroundings, giving information both on the temporal (accumulation) and the spatial axis. Plants, in turn, are sessile, but expand their roots both laterally and in depth, absorbing pollutants from successive strata.

The application of Comet Assay to earthworms, and consequently the use of such extraordinary prospectors as sentinels for the presence of genotoxicants in soil, started in the nineties of the last century (Singh et al., 1988; Verschaeve and Gilles, 1995; Salagovic et al., 1996), and since then has been extensively revised (Cotelle and Férard, 1999; Espinosa-Reyes et al., 2010; Liu et al., 2010; Atli Şekeroglu et al., 2011; Lionetto et al., 2012; Andem et al., 2013; Vernile et al., 2013; Fujita et al., 2014; Vasseur and Bonnard, 2014; Zhang et al., 2014). Several earthworms comparative studies have been performed (Vasseur and Bonnard, 2014). *Eisenia fetida* and *Aporrectodea caliginosa* showed an equivalent sensitivity, as assessed by Comet Assay (Klobučar et al., 2011). Fourie et al. (2007) compared the sensitivity of five earthworm species (*Amynthas diffringens, A. caliginosa, E. fetida, Dendrodrilus rubidus* and *Microchaetus benhami*) to Cd genotoxicity after a 48 h-exposure. *E. fetida* presented the highest percent of DNA in tail and was the second most sensitive species after *D. rubidus*, which showed the highest increase in DNA breaks compared with the control.

Plants are also specially well-fitted for ecotoxicological assessment of soils, including genotoxicity. The Comet Assay may be performed in different organs (nucleus of roots cells or leaf cells), and combined, when suitable, with growth tests (Grant, 1994; Sandhu et al., 1994; Gopalan, 1999; Ma, 1999; Sadowska et al., 2001; Ma et al., 2005). However, cell lysis and release from plant cells is challenging and require special adaptations to the protocol (such as mechanical extraction of nuclei or protoplast production), which may be tissue– and species–dependent (see Costa et al., 2012a and references therein). In general, the Comet Assay in plants is far from being as common and widespread as in animals.

Genotoxicants in the terrestrial compartment have also been tracked by means of Comet Assay using vertebrates as sentinel species, particularly avian and rodents. The ecological disaster occurred in April 1998 in the mines of Aznalcóllar, consisting in a massive toxic spill of acid waste containing metals, threatened the wildlife in the Doñana National Park in SW Spain. The presence of DNA damage was studied along 4 years by means of Comet Assay in white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) (Pastor et al., 2001, 2004; Baos et al., 2006). Results indicate that the exposed birds had a significantly increased level of genotoxic damage compared with control animals from non-contaminated locations, that the toxic spill still appears to be affecting the wildlife 4 years after the mining disaster and that attempts at cleaning up the waste have proved ineffective based on DNA damage detection. A study to determine DNA damage in blood cells of barn swallows (*Hirundo rustica*) inhabiting the Chernobyl region was carried out, to evaluate whether chronic exposure to low-level radioactive contamination continues to induce genetic damage in free-living populations of animals. The results showed that Comet values in barn swallows living in areas surrounding Chernobyl are still increased when compared to swallows sampled at low-level sites, even 20 years after the accident at the Chernobyl nuclear power plant (Bonisoli-Alquati et al., 2010).

Rodent species have been used as sentinels of eco-genotoxicity in a variety of scenarios. The European wood mice (*Apodemus sylvaticus*) is a ubiquitous, abundant species which has been studied to assess the effects of dumping sites (Delgado et al., 2000), urban or traffic pollution (Borràs and Nadal, 2004) or the surroundings of an abandoned uranium mining site (Lourenço et al., 2013). In all these cases, the combination of Comet Assay and wood mice proved to be a sensitive and reliable tool for the detection of the exposure to environmental genotoxicants. The yellow-necked wood mouse (*Apodemus flavicollis*) is a closely–related species inhabiting the regions of central and northern Europe. A study was performed in different protected areas of the Strandzha National Park in Bulgaria in 2010 and 2011. An increase in the Comet Assay parameters in the analyzed individuals of yellow-necked mouse from the Sredoka protected area was established. Those results indicated that there was genetic damage in some mice populations as a consequence of chronic contamination (Mitkovska et al., 2012). The Algerian mouse (*Mus spretus*) is a similar species, more frequent in south-Europe. This species has been used in different studies, however. A comparison was done between mice living in an industrial area in the neighborhood of Huelva city, SW Spain, and in a natural area (Doñana National Park). Results suggest that Comet Assay in wild mice can be used as a valuable tool in pollution monitoring (Mateos et al., 2008). Genotoxicity monitoring using the Comet Assay on peripheral blood leukocytes of the Algerian mouse was carried out in Doñana Park (Spain), after the environmental disaster of the Aznalcollar pyrite mine in 1998. The mice were sampled in different areas 6 months after the ecological disaster and again 1 year later. Results showed that in 1998 Comet parameters were increased in all the areas examined, whereas a significant decrease in the values was observed in the 1999 samples, which were collected in a riverside area subject to tide flows (Festa et al., 2003).

Wild individuals of *Rattus rattus* and *Mus musculus* have also been assessed for DNA damage by the Comet Assay. A study was conducted in a coal mining area of the Municipio de Puerto Libertador, Colombia. Animals from two areas in the coal mining zone and a control area were investigated. The results showed evidence that exposure to coal results in elevated primary DNA lesions in blood cells of rodents (León et al., 2007). Meadow voles (*Microtus pennsylvanicus*) have been used to measure the effects of pesticide exposure in golf courses of the Ottawa/Gatineau region of Canada (Knopper et al., 2005). *Ctenomys torquatus* is a South-American species which was used for biomonitoring in the coal region of Rio Grande do Sul (Brazil). The results of this Comet Assay study indicate that coal and by-products not only

induce DNA damage in blood cells, but also in other tissues, mainly liver, kidney, and lung (da Silva et al., 2000a,b).

It is also worth to note how a multi-trophic level approach may be applied to assess the impact of toxicity on a given ecosystem. A recent example is the assessment of the effect of radioactive materials released in 2011 during the accident at Japan's Fukushima nuclear power plant on wildlife. The effects of exposure to environmental radiation were studied by means of Comet Assay in wild boars (*Sus scrofa leucomystax*) and earthworms (*Megascolecidae*). Regions with low (0.28 μSv/h) and high (2.85 μSv/h) levels of atmospheric radiation were compared. The authors constructed a model food web featuring the wild boar as the top predator, and measured the radioactivity levels in soil, plant material, earthworms, and wild boar. The extent of DNA damage in wild boars did not differ significantly between animals captured in the two regions, but earthworms from the "high-dose" region had a significantly greater extent of DNA damage than did those from the "low-dose" region (Fujita et al., 2014).

A Methodological Overview

Amphibians

Over the years, the Comet Assay protocol has undergone some alterations; however there is no clear evolution or tendency (see **Table 1**). Regarding the lysis buffer, in the first papers published by Ralph et al. (1996) and Ralph and Petras (1998b) and also by Clements et al. (1997) no detergent (e.g., Triton X-100) nor DMSO were added to the stock solution. Later, in 1997 and 1998, Ralph and Petras (1997, 1998a), added these components to the lysis buffer, which made it very similar to the buffers commonly used nowadays in most of the studies published. Ever since, in most of the studies, the buffer includes these two components, with few exceptions (Chemeris et al., 2004; Valencia et al., 2011; Zhang et al., 2012; Meza-Joya et al., 2013). Additionally some variations are also found in the composition of the lysis buffers, such as the inclusion or exclusion of some commonly used reagents like, for example, the replacement of sodium sarcosinate with SDS as detergent. However, in two particular studies performed by Valencia et al. (2011) and also Meza-Joya et al. (2013), a different lysis buffer and lysis protocol is used. These authors exposed the cells to a lysing solution containing proteinase K and calcium chloride, before the cells were mixed with the agarose and spread out on slides. This protocol was used in blood cells from *Eleutherodactylus johnstonei* to overcome the problem of lysing those cells, which were seemingly resistant to the lysis treatments commonly performed. Thus, this appears to be an important factor to consider in future studies with similar species. Regarding lysis itself, it is usually performed under alkaline conditions, using time intervals varying from 25 min to a maximum of 1 week. Until 2005, lysis was usually performed at room temperature, however, from 2006 until now it is generally conducted at 4°C, which is in agreement to the guidelines published by Azqueta and Collins (2013). The low melting point agarose concentration it is usually 0.5%, but it varies from 0.4 to <1%, which limits the comparison of the results obtained in the various studies, since it directly affects DNA migration. Accordingly, the higher the agarose concentration, the lower the % tail DNA (Azqueta and Collins, 2013). Denaturation is generally conducted in alkaline conditions (pH > 13), from 5 min to 40 min which, once again, limits the comparison between studies, since it also affects DNA migration. As referred by Azqueta and Collins (2013), the higher the incubation period the higher the % tail DNA. Regarding electrophoresis, voltage can vary between 18 and 27 V, generally at 300 mA, from 4 to 50 min. However, not all the studies refer the voltage gradient used (V/cm), and therefore a comparison between studies is still a limitation. Generally, variation between protocols, mainly regarding agarose concentration, denaturation and electrophoresis conditions, denotes lack of standardization, compromising direct comparisons between studies.

Piscine Models

The wide variety of fish species addressed, tissues sampled, and experimental approaches adopted have led to a profusion of adaptations to the Comet Assay protocol (see **Table 2**). To date, no standardized Comet Assay procedures exist for environmental studies involving fish. In addition, a standardization of sampling protocols when using laboratory exposed or both transplanted and wild specimens in biomonitoring studies is required (Frenzilli et al., 2009).

The Comet Assay adopted in different contexts has proved to be also valuable in the elucidation of the mechanisms of genotoxicity and DNA repair. In this direction, the implementation of a protocol with an extra step where nucleoids are incubated with DNA lesion-specific repair endonucleases has added greatly to the value of the Comet Assay (Azqueta and Collins, 2013), namely on the specific detection of oxidized bases and thus, identifying oxidative DNA damage as a harmful process underlying the genomic integrity loss. The use of endonuclease III (thymine glycol DNA glycosylase-Endo III) was initially proposed by Collins et al. (1993) to specifically target oxidized pyrimidines, while formamidopyrimidine DNA glycosylase (Fpg) was firstly adopted by Dusinska and Collins (1996) to signal oxidized purines. The adoption of this improved procedure in the field of environmental genotoxicology using piscine models took almost one decade, since, to the authors' knowledge, it was applied for the first time in 2003 (Akcha et al., 2003). This enzyme-modified assay has attracted particular attention in the last years, being applied either in whole organism (Tomasello et al., 2012), involving different tissues (blood, liver, and gill) (Aniagu et al., 2006), or cell line (Kienzler et al., 2012) testing. It was concluded that the scoring of the DNA damage encompassing oxidatively induced breaks increases sensitivity (Tomasello et al., 2012) and reduces the possibility of false negative results (Guilherme et al., 2012a) when compared to the standard Comet Assay. This approach can be particularly informative when the additional breaks corresponding to net enzyme-sensitive sites are shown (Guilherme et al., 2012a). In the light of these positive outcomes, it seems clear that this specific tool has been underexploited.

Another technical development concerns the adoption of Comet Assay to evaluate the DNA repair ability of a specific tissue (Collins et al., 2001), namely through the *in vitro* assays

TABLE 1 | Summary of the methodological developments on amphibians.

Experimental model	Contaminants tested	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
					pH	Temp. (°C)	Duration (h)	pH	Duration	Voltage	Duration	
Lithobates clamitans *Anaxyrus americanus* *Lithobates catesbeianus* (tadpoles)	MMS Atrazine Metalochlor Glyphosate Metribuzin 2,4-D amine	Blood	0.4 0.4 0.5	Buffer A: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Na - sarcosinate	10	RT	2	13	15 min	25 V (265–270 mA)	20 min (37°C)	Ralph et al., 1996; Clements et al., 1997; Ralph and Petras, 1998b
Anaxyrus americanus *Lithobates clamitans* *Lithobates pipiens* (tadpoles)	Several pollutants	Blood	0.4	Buffer B: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Na - sarcosinate, 1% Triton X-100	10	RT	2	13	15 min	25 V (265–270 mA)	20 min (37°C)	Ralph and Petras, 1997, 1998a
Euphlyctis hexadactylus (tadpoles)	Sulfonated dyes	Blood	0.75	Buffer C: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100	10	RT	2	13	25 min	20 V (300 mA)	20 min	Rajaguru et al., 2001
Xenopus laevis (adults)	High peak-power pulsed electromagnetic field	Blood	0.5	Buffer D: 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, 100 mM Na_2EDTA	10	22	25 min	13	20 min	1.9 V/cm	20 min (4° C)	Chemeris et al., 2004
Pelophylax nigromaculatus (adults)	Imidacloprid RH-5849	Blood	0.5	Buffer B	10	RT	1	13	25 min	25 V (150 mA)	50 min	Feng et al., 2004
Xenopus laevis *Pleurodeles waltl* (tadpoles)	Several pollutants Benzo(*a*)pyrene EMS MMS Captan	Blood	0.5	Buffer C	10	RT	1	13	20 min (4°C)	20V (300 mA)	20 min (Xenopus) 30 min (*Pleurodeles*) (4° C)	Mouchet et al., 2005a,b, 2006a,b
Strauchbufo raddei (tadpoles and adults)	Acetochlor Petrochemical contaminants	Blood Hepatocytes	0.6 0.5	Buffer B	10	4	1	13	20 min (4°C)	25 V (300 mA)	20 min (4° C)	Liu et al., 2006; Huang et al., 2007
Xenopus laevis *Xenopus tropicalis* (adults)	Bleomycin	Splenic lymphocytes	nm	Buffer B	10	4	30 min	10	30 min (RT)	24 V	4 min (TBE; RT)	Banner et al., 2007

(Continued)

TABLE 1 | Continued

Experimental model	Contaminants tested	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
					pH	Temp. (°C)	Duration (h)	pH	Duration	Voltage	Duration	
Bufo gargarizans (tadpoles)	Acetochlor, Butachlor, Chlorimuron-ethyl Paraquat Chlorpyrifos	Blood Hepatocytes	<0.8	Buffer B	10	4	2	13	30 min (4°C)	18 V (300 mA)	20 min (4°C)	Yin et al., 2008, 2009
Pelophylax nigromaculatus (adults)	Lead	Testicular cells	0.3	Buffer E: 2.5 M NaCl, 10mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% SDS, 1% Triton X-100, pH 10	10	4	2–4	10	30 min (4°C)	22 V (220 mA)	30 min (4°C)	Wang and Jia, 2009
Pelophylax lessonae (adults)	Several pollutants	Blood	0.5	Buffer C	10	4	1	13	nm	20 V (300 mA)	20 min (4°C)	Maselli et al., 2010
Fejervarya limnocharis (tadpoles)	Butachlor	Blood	0.9	Buffer B	10	RT	1	13	20 min (RT)	23 V (300 mA)	25 min (RT)	Liu et al., 2011
Eleutherodactylus johnstonei (adults)	Bleomycin 4-nitro-quinoline-1-oxide Glyphosate	Blood	0.7	Buffer F: 50 mM Tris, 10 mM CaCl2, 0.04 g/mL proteinase K	8	6 ± 2	10 min	13	25 min	25 V (300 mA, 0.90 V/cm)	30 min (6 ± 2°C)	Valencia et al., 2011; Meza-Joya et al., 2013
Fejervarya limnocharis (adults)	Cadmium	Testicular cells	<1	Buffer A	10	RT	2	13	30 min (4°C)	22 V (220 mA)	30 min (4°C)	Zhang et al., 2012
Hypsiboas faber (adults)	Heavy metal pollution	Blood	0.7	Buffer C	10	4	1	13	20 min	25 V (300 mA, 0.90 V/cm)	20 min	Zocche et al., 2013
Rana temporaria (adults)	–	Sperm cells	0.5	Buffer B	10	4	1	13	20 min	27 V (260–270 mA, 0.2 V/mm)	? (4°C)	Shishova et al., 2013
Rhinella marina (adults)	POPs	Blood	0.5	Buffer C	13	4	1 week	13	5 min	25 V (300 mA)	10 min	Gonzalez-Mille et al., 2013
Pelophylax ridibundus (adults)	Several pollutants	Blood	0.5	Buffer B	10	4	1	13	40 min	25 V (300 mA)	25 min	Erismis et al., 2013
Duttaphrynus stomaticus (adults)	Chlorpyrifos	Blood	0.5	Buffer C	10	4	2	13	25 min	2 5V (300 mA, 0.73 V/cm)	25 min	Ismail et al., 2014

RT, room temperature.

TABLE 2 | Summary of the methodological developments on piscine models.

Experimental model	Contaminants tested	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
					pH	Temp (°C)	Duration (h)	pH	Duration	Voltage	Duration	
Ameiurus nebulosus and *Cyprinus carpio*	Environmental exposure to PAHs and PCBs; Lab exposure to cyclophosphamide	Blood	0.5	2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Na sarcosinate	10	RT	2	nm	15 min	25 V (265–270 mA)	20 min (3°C; dark)	Pandrangi et al., 1995
Psetta maximus	Ethyl methane-sulphonate (EMS)	Blood, gill, liver and kidney	0.8	2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% N-lauroylsarcosine, 1% Triton-X 100, 10% DMSO	nm	nm	1–2	>13	20–40 min	0.4–0.7 V/cm (300 mA)	10–20 min	Belpaeme et al., 1998
Oncorhyncus mykiss, Ictalurus punctatus	Aflatoxin B1	Blood, liver, kidney	0.75	2.5 M NaCl, 10 mM Tris-base, 0.1% sodium sarcosinate	10	RT	2	>13	15 min	20 V (300 mA)	25 min	Abd-Allah et al., 1999
Pholis gunnellus	Environmental exposure to PAHs and metals	Blood	1.0	2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton-X 100, 10% DMSO	10	4	1	12.3	30 min	25 V (300 mA)	15 min	Bombail et al., 2001
Limanda limanda	Environmental exposure to PAHs and PCBs	Blood	0.5	NaCl 2.5M, Na2 EDTA 0.1M, Tris base 0.01M, N-sarcosinate 1%, DMSO 10%, Triton X-100 1%	10	RT	1	13	15 min	23 V (390 mA)	20 min	Akcha et al., 2003
Oreochromis niloticus	Benzocaine	Blood	0.5	2.5M NaCl2, 100 mM Na2EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO	10	4	1	>13	5–40 min	0.66 V/cm	5–40 min	de Miranda Cabral Gontijo et al., 2003
Danio rerio	4-nitroquinoline-1-oxide	Hepatocytes and gill cells	0.7	2.5M NaCl, 10 mM Tris, 100 mM EDTA, 1% Na-sarcosinate, 10% DMSO, 1% Triton X-100	10	nm	nm	nm	20 min	25 V (300 mA)	20 min	Diekmann et al., 2004b
Petromyzon marinus	Bisazir	Spermatozoa	nm	2.5M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton X-100, 1% DMSO	nm	4	1	>13	1 h	25 V (300 mM)	30 min	Ciereszko et al., 2005

(Continued)

TABLE 2 | Continued

Experimental model	Contaminants tested	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
					pH	Temp (°C)	Duration (h)	pH	Duration	Voltage	Duration	
Geophagus brasiliensis, Cichla temensis, Hoplias malabaricus, Astyanax bimaculatus lacustres, Oreochromis niloticus, Cyprinus carpio, Steindachnerina insculpita	Eutrophication	Blood	nm	NaCl 2.5 M; EDTA 100 mM; Tris 10 mM; N-laurolyl-sarcosine 1%; Triton-X 1%; DMSOn 10%	10	4	30 min	13	30 min	25 V (350 mM)	30 min (4°C; dark)	Grisólia et al., 2009
Oncorhynchus mykiss	Cryopreservation	Spermatozoa	nm	2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% Triton-X, 1% lauroyl sarcosine sodium salt, 4 mM lithium diiodosalicylate	8	4	1	12	20 min	25 V (300 mM)	10 min	Pérez-Cerezales et al., 2010
Oncorhynchus mykiss, Danio rerio	Trenbolone	Cell lines	0.7	100 mM EDTA, 2.5 M NaCl, 1% Triton X-100, 10% DMSO	13	4	1.5	nm	nm	25 V (310 mM)	nm	Boettcher et al., 2011
Anguilla anguilla	Glyphosate-based herbicide	Blood and liver	1.0	2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100	10	4	1	>13	20 min	25 V (300 mM)	15 min	Guilherme et al., 2012b
Ictalurus punctatus	Water and sediment from gypsum mining area	Ovary cell line	nm	2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, 1% Na-sarcosinate, 1% Triton X-100, 10% DMSO	10	nm	nm	13	20 min	1 V/cm (300 mA)	20 min	Ternjej et al., 2013
Catla catla, *Labeo rohita*	Silver nanoparticles	Heart and gill cell lines	0.9	2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% DMSO, 1% Triton X-100	10	4	1	>13	20 min	25 V (300 mM)	20 min	Taju et al., 2014

nm, not mentioned; RT, room temperature.

for nucleotide excision repair (NER) and base excision repair (BER). For these assays, a DNA substrate containing specific lesions is incubated with an extract prepared from the tissue to test. The accumulation of breaks due to the incubation with that extract is a measure of DNA repair activity in the tissue (Azqueta et al., 2013). The few studies published using this type of assay include the detection of tissue-specificities of BER activity in *Xiphophorus* species, showing that brain possesses higher BER activity than gill and liver (Walter et al., 2001). The other available publications resulted from the work of the same research group and concern the application of BER (Kienzler et al., 2013a) and NER (Kienzler et al., 2013b) assays in fish cultured cells. Though the previous publications recommend the adoption of these DNA repair biomarkers as a complement the more classical genotoxicity endpoints (Kienzler et al., 2013a), their application has been clearly underestimated.

Blood has been, undoubtedly, the preferred tissue to perform Comet Assay in fish (e.g., Guilherme et al., 2010; Lourenço et al., 2010; Ternjej et al., 2010), mainly due to the easy sampling and availability of dissociated cells, a critical factor. All fish blood cells are nucleated which also represents an important practical advantage (comparing to mammals) for the assessment of genomic integrity. Nevertheless, other somatic tissues like liver, kidney and gills have been also frequently addressed (Guilherme et al., 2012b; Kumar et al., 2013; Velma and Tchounwou, 2013), as well as germ cells (Pérez-Cerezales et al., 2010). It is recognized that DNA strand breakage can be tissue- and cell-type-specific (Pandey et al., 2006). Hence, it is improbable that blood cells can reflect the type and extent of DNA damage occurring in other cell types. The choice of blood has been mainly determined by practical/technical reasons and rarely relied on the knowledge of a comparative performance with other target tissues. It has been stated that circulating cells are less sensitive, when compared to other types of cells (Frenzilli et al., 2009), but this is not a consensual assumption. As an example, a comparison between DNA damage in gill, kidney and blood tissues of *Therapon jarbua* following an exposure to mercuric chloride indicated the following order in terms of sensitivity: gill > kidney > blood cells (Nagarani et al., 2012). Guilherme et al. (2012b) stated that DNA damage in liver returned faster to the control level comparing to gills, which was regarded as an indication of a better adaptive behavior of hepatic cells, probably related with a higher capacity to maintain the genomic stability by detecting and repairing damaged DNA.

Bivalves and Other Molluscs

Haemocytes are the most common target for genotoxicity assessment *in vivo* and *in vitro* in bivalves and gastropods (see **Table 3**). Although collection requires some skill, obtaining haemocytes from bivalve adductor muscles or haemocoel (e.g., pericardial) in bivalves and gastropods is proved to be feasible and able to yield cells apt for the Comet Assay in both number and quality. Still, it has been noted, concerning terrestrial snails, that broken or detached epiphragms may cause significant dehydration of tissues, hampering collection of haemolymph (Angeletti et al., 2013). Altogether, it is likely that haemolymph collection needs to be properly set and tested for each target organism. Gills have also been successfully employed since cell resuspension is easy enough to be assisted by gentle tissue splicing and "soft–pipetting" followed by low–speed centrifuging (≈2000 g) to remove debris and dead cells, without the need for treatment with collagenase (see Martins et al., 2012). Still, it has been shown that the baseline DNA strand breakage may greatly differ between organs.

The molluscan digestive gland, the analogous of the vertebrate liver and therefore of high relevance in toxicological studies, was shown to yield levels of single strand breakage likely too high (from autolytic processes) for a valid application of the Comet Assay without proper cell sorting and viability check (refer to Raimundo et al., 2010, in a study with the cephalopod *Octopus vulgaris* and Hartl et al., 2004 with the clam *Ruditapes philippinarum*). Recent advances have also shown the feasibility of obtaining adequate cultures of molluscan cells for *in vitro* studies using the Comet Assay (Michel and Vincent-Hubert, 2012) and even the possibility to cryopreserve mussel haemocytes (Kwok et al., 2013). Altogether, these advances certainly contribute to standardize the Comet Assay in biomonitoring and genotoxicity testing with bivalves and other molluscs.

Terrestrial Organisms

The Comet Assay in earthworms is performed on the small cells which constitute the most abundant class among the cellular population of the coelomic fluid, and that are the homologous, in worms, of vertebrate leucocytes. Cells are collected according to Eyambe et al. (1991), or by means of electric or ultrasonic stimulation. *Eisenia foetida* (*andrei*) is the most commonly used species, owing to the fact of being the one recommended by international guidelines for lethality and reproduction ecotoxicology studies; however, other species have been used, as for instance *A. caliginosa* (Klobučar et al., 2011), *Lumbricus terrestris*, *L. rubellus* (Spurgeon et al., 2003), *D. rubidus* and *M. benhami* (Fourie et al., 2007), among others (Vasseur and Bonnard, 2014).

Performing the Comet Assay in vegetal cells, however, present some particular difficulties (Gichner and Plewa, 1998). The rigid cellulose cell walls prevent DNA from leaving the cell, and are not easily eliminated with the usual alkaline treatment; so, nuclei isolation from tissues is necessary as a first step. However, the isolation procedure (either mechanical or chemical) may produce some degree of nuclear disruption, which could in some cases constitute a serious handicap. On the other hand, the high concentration of pigments and metabolites present in photosynthetic tissues (as leaves) tends to cause further damage to the isolated nuclei. To avoid this concern, root apical tissue is often preferred, but, in this case, the high rate of cell division may in turn be a problem. To reproducibly perform Comet Assay in leaves, some modifications to the standard procedure have been proposed, which includes a centrifugation through sucrose cushion, to eliminate disrupted nuclei and secure a higher fraction of undamaged nuclei (Peycheva et al., 2011). Recently, protocols have been developed to perform the Comet Assay in tree cell cultures from protoplasts following failure to obtain nude nuclei by the most common mechanical processes (Costa et al., 2012a) In spite of these difficulties, the Comet Assay has been

TABLE 3 | Summary of the methodological developments on Bivalves and other Molluscs.

Experimental model	Contaminants tested	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
					pH	Temp. (°C)	Duration (h)	pH	Duration	Voltage	Duration	
Mytilus edulis; Ruditapes decussatus (Bivalves)	Environmental exposure to PAHs, PCBs and metals;	Gills	1	Buffer A: 2.64% NaCl (w/v), 3.72% EDTA (w/v), 5 mM TRIS, 10% DMSO (v/v), 1% Triton-X 100	10	4	1	13	40 min	25 v	30 min	Martins et al., 2012, 2013
Octopus vulgaris (Cephalopods)	Heavy metal pollution	Gills, Digestive gland; "Kidneys"; Gonads	1	Buffer A	10	4	1	13	40 min	25 v	30 min	Raimundo et al., 2010
Patella vulgata (Gastropods)	Environmental exposure to PAHs	Haemolymph	1	n/m	10	5	1	13	45 min	25 V	30 min	Lewis et al., 2010
Littorina littorea (Gastropods)	Environmental exposure to OCP (organochlorine pesticides), PCBS, PAHs	Haemolymph	1	n/m	10	5	1	13	45 min	25 V	30 min	Noventa et al., 2011

nm, not mentioned.

successfully used in recent years to test the effects of Cr(VI) in *Pisum sativum* (Rodriguez et al., 2011), of Chlorfenvinphos and fenbuconazole in *Allium cepa* (Türkoğlu, 2012), cadmium-zinc (Cd-Zn) interactions in tobacco plant (Tkalec et al., 2014) or to demonstrate the correlation between the occurrence of B chromosomes and the DNA damage that is induced by the chemical mutagen, maleic hydrazide (MH), in *Crepis capillaris* plants (Kwasniewska and Mikolajczyk, 2014), among others. A recent revision (Ventura et al., 2013) is available.

There are a variety of working protocols of the comet assay for both birds and mammals (see **Table 4**). Circulating lymphocytes are used mainly as the test cell type because of its available and because it can be a non-invasive method of extracting sample (Azqueta and Collins, 2013). As described previously, the use of lesion-specific repair endonucleases has been employed in studies with in terrestrial organisms. This aspect brings to the Comet Assay a very interesting added value for targeting routes that are acting during exposure.

Correlations with Other Biomarkers

Amphibians

The combination of Comet Assay, to detect DNA strand breaks, with the evaluation of other biomarkers to determine the effects of contaminants in exposed organisms has been performed in many studies. Some of those studies show a positive correlation between the results given by the Comet Assay and other biomarkers. For instance, in the studies performed by Mouchet et al. (2005a,b, 2006a,b), a positive correlation between DNA strand breaks detection and micronucleus induction was observed most of the times. This result was expected since the Comet Assay measures primary DNA damages and the micronucleus test reflects irreparable lesions that result from the non-repaired or inappropriately repaired primary DNA damages, which are likely to be inherited by subsequent generations of cells. In another study Liu et al. (2006) investigated the role of reactive oxygen species (ROS) in the herbicide acetochlor-induced DNA damage on *Strauchbufo raddei* tadpole liver and the results showed a positive correlation between DNA damage and malondialdehyde (MDA) formation and a negative correlation between DNA damage and total antioxidant capability. This result showed that the herbicide acetochlor induce DNA damage through the formation of ROS. Zhang et al. (2012) conducted a study to evaluate cadmium-induced oxidative stress and apoptosis in the testis of frog *Fejervarya limnocharis,* which also showed a positive correlation between DNA damage, lipid peroxides and ROS formation and gluthatione determination, showing the role of oxidative stress to damage DNA of these cells. These studies show the importance of the inclusion of the Comet Assay in a battery of tests that contribute to determine the chain of events leading to the effects observed and to determine the type of damages to DNA.

Piscine Models

As a sign of maturity, in the last years a particular attention has been devoted to the interference of non-contamination related factors (biotic and abiotic) with the genotoxicity expression.

TABLE 4 | Summary of the methodological developments on Terrestrial organisms.

Experimental model	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
				pH	Temp. (°C)	Duration (h)	pH	Duration (min)	Voltage	Duration	
Aporrectodea caliginosa; Amynthas diffringens; Dendrodrilus rubidus; Eisenia fetida; Microchaetus benhami (earthworms)	Coelomocytes	0.5	Buffer A: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 0.2 M NaOH, 1% Triton X-100	10	4°C	15	13	20 min	35 V (300 mA)	10 min	Fourie et al., 2007
Eisenia fetida; Eisenia sp. (earthworms)	Coelomocytes	n/m	Buffer B:2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% DMSO, 1% triton X-100	10	4°C	24	13	5 min	25 V (300 mA)	5 min (4°C)	Espinosa-Reyes et al., 2010
Aporrectodea caliginosa; Eisenia fetida (earthworms)	Coelomocytes	0.8	Buffer B	10	4°C	1	13	15 min	35 V (300 mA)	20 min (4°C)	Klobučar et al., 2011
Eisenia andrei Bouché (earthworms)	Coelomocytes	1.0	Buffer C: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% triton X-100, 0,2 NaOH	10	4°C	Overnight	13	25 min (RT)	0.74 V/cm	30 min (RT)	Vernile et al., 2013
Eisenia fetida (earthworms)	Coelomocytes	n/m	Buffer B	10	4°C	1, 5	n/m	20 min (RT)	25 V (300 mA)	20 min	Saez et al., 2014
Ciconia ciconia; Milvus migrans (birds)	Blood	0.7	Buffer D: 2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% sodim sarcosinate, 1% Triton X-100	10	4°C	1	12.8	20 min (4°C)	1.6 V/cm (300 mA)	20 min (4°C)	Pastor et al., 2004
Hirundo rustica (birds)	Blood	1.0	Buffer E: Lysis Solution, Trevigen, 10% DMSO	10	4°C	1	12.1	45 min	25 V (250 mA)	10 min	Bonisoli-Alquati et al., 2010
Ctenomys torquatus (rodents)	Blood	0.75	Buffer B	10		1–2 weeks	12.6	31 min	25 V (300 mA)	30 min	da Silva et al., 2000a,b
Apodemus sylvaticus (rodents)	Blood	0.7	Buffer C	10	n/m	n/m	13	20 min	25 V (300 mA)	15 min	Festa et al., 2003
Microtus pennsylvanicus (rodents)	Blood	0.75	Buffer F: 2.5 M NaCl, 10 mM Na2EDTA, 10 mM Tris Base, 1% n-lauryl sarcosinate, 1% Triton X-100	10	4°C	Overnight	13.1	30 min	27 V	20 min	Knopper et al., 2005
Rattus rattus; Mus musculus (rodents)	Blood	n/m	Buffer B	10	4°C	2 weeks	13	30 min (4°C)	25 V (300 mA)	30 min	León et al., 2007

(Continued)

TABLE 4 | Continued

Experimental model	Cell type/tissue	Agarose (%)	Lysis buffer composition	Lysis conditions			Denaturation conditions		Electroforesis conditions		References
				pH	Temp. (°C)	Duration (h)	pH	Duration (min)	Voltage	Duration	
Mus spretus (rodents)	Blood	0.7	Buffer C	12	4°C	1	12.8	20 min	1 V/cm	20 min	Mateos et al., 2008
Apodemus sylvaticus (rodents)	Blood	0.5	Buffer B	10	4°C	1	n/m	15 min	0.7 V/cm (300 mA)	10 min	Lourenço et al., 2013
Nicotiana tabacum (plants)	Leaves	0.75	Buffer C	10		1	13	20 min	25 V (300 mA)	20 min (4°C)	Gichner and Plewa, 1998
Allium cepa; Nicotiana tabacum (plants)	Roots, leaves	n/m	n/a	n/a	n/a	n/a	13	15 min	26 V (300 mA)	20 min (4°C)	Ghosh et al., 2010
Allium cepa (plants)	Roots	n/m	n/a	n/a	n/a	n/a	13	12 min	0.75 V/cm (300 mA)	15 min (4°C)	Panda et al., 2011
Pisum sativum L (plants)	Leaves	1.4	n/a*	n/a*	n/a*	n/a*	12.6	30 min (4°C)	0.45 V/cm	10 min	Peycheva et al., 2011
Pisum sativum L (plants)	Roots, leaves	1	n/a	n/a	n/a	n/a	13	15 min	0.74 V/cm	15 min	Rodriguez et al., 2011
Crepis capillaris (plants)	Leaves	1	n/a	n/a	n/a	n/a	13	15 min	15 V (340 mA)	15 min (4°C)	Kwasniewska and Mikolajczyk, 2014
Allium cepa (plants)	Roots	0.75	n/a	n/a	n/a	n/a	13	12 min	0.75 V/cm (300 mA)	15 min (4°C)	Pakrashi et al., 2014
Nicotiana tabacum L (plants)	Seeds, roots, leaves	1	n/a	n/a	n/a	n/a	13	10 min	0.8 V/cm (300 mA)	20 min	Tkalec et al., 2014
Picea abies (plants)	*Protoplasts from embryogenic cultures*	1	Buffer B	10	4	1.5	13	40	25 V	30 min	Costa et al., 2012a

*n/m, not mentioned; n/a, not applicable (For isolation of nuclei, tissues are treated with 400 mM Tris buffer pH 7.5, and then finely and gently sliced with a razor blade); * nuclei isolation was performed by: (1) cutting of the tissue, (2) homogenizing in phosphate buffered saline (PBS), (3) adding protease inhibitor phenylmethylsulphonyl fluoride (PMSF), (4) centrifuging and (5) re-suspending in PBS.*

This is a critical knowledge to allow a correct assessment of the contribution of chemical contamination to the DNA damage measured. In this direction, hypoxia, and hyperoxia, known as important stressors in the aquatic environment, were tested in *Cyprinus carpio*, revealing that both conditions increase oxidative DNA damage (approximately 25% compared to normoxic conditions) (Mustafa et al., 2011). Another study demonstrated that acute extreme exercise results in oxidative DNA damage in *Leuciscus cephalus*, suggesting that fish living in fast flowing and polluted waters are at increased risk (Aniagu et al., 2006). The effects of age, gender, and sampling period were also investigated (Akcha et al., 2004). In adult fish (*Limanda limanda*), DNA breaks were higher in males than in females, whereas the opposite trend was observed for juveniles. Regardless of gender, the extent of DNA damage was higher in the adult comparing to juvenile fish. It was also suggested that the formation of DNA lesions can be modulated by seasonal variables, namely those related to variations in lipid content, biotransformation activity and/or to spawning cycles (Akcha et al., 2004). It was hypothesized that anesthesia used before tissue sampling can have confounding influences on the DNA integrity evaluation. Still, Nile tilapia exposed to benzocaine showed that this anesthetic does not affect Comet Assay results (de Miranda Cabral Gontijo et al., 2003).

The assumption that the Comet Assay can be successfully applied to monitor effects of environmental disturbances emerged unanimously from the majority of fish studies using this technique (e.g., Ciereszko et al., 2005; Srut et al., 2010). Tough a more skeptical perspective can detect in this unanimity a self-worth and self-legitimation positioning, it is also clear that it represents a strengthening of the goodness of the assertion. It has been suggested that the ecotoxicological consequences of a genomic instability and its correlation with DNA breaks measured by the Comet Assay deserves a special attention (Jha, 2008). To gain ecological relevance, a mechanistic association between genotoxic stress and effects at higher biological levels should be identified, contributing to predict deleterious effects mainly at population level (e.g., abundance and reproduction impairments). The controversy whether adverse effects of anthropogenic genotoxicants can be associated to the decline of fish populations has been the leitmotiv for some recent studies. A complete life-cycle test was carried out with zebrafish (*Danio rerio*) and the model genotoxicant (4-nitroquinoline-1-oxide) seeking for a causal linkage between genotoxic effects and ecotoxicological risk (Diekmann et al., 2004a,b). It was observed a reduction of egg production, which would have led to fish extinction according to a mathematical simulation (Diekmann et al., 2004a), concomitantly with DNA damage induction (Diekmann et al., 2004b). However, this study failed on demonstrating a direct evidence that genotoxicity is functionally related to reduced egg production (Diekmann et al., 2004a). The assessment of the consequences of germ cell DNA damage on progeny outcomes has been regarded as a strategy to signal potential long-term effects of aquatic genotoxicants in fish, since genetic damage in such cells, if unrepaired or misrepaired, can be passed on to future generations (Devaux et al., 2011). In this direction, it was demonstrated a positive correlation between the DNA damage in sperm from parental fish (*Salmo trutta* and *Salvelinus alpinus*) exposed to the alkylating genotoxicant model methyl methanesulfonate and the incidence of skeletal abnormalities in the offspring, clearly suggesting that DNA damage had been inherited (Devaux et al., 2011). In a subsequent study, spermatozoa of *Gasterosteus aculeatus* were exposed *ex vivo* to MMS before *in vitro* fertilization and a relationship between abnormal embryo development in the progeny and sperm DNA damage was demonstrated (Santos et al., 2013). It was also revealed that sperm of *Oncorhynchus mykiss* maintains its ability to fertilize in spite of having DNA damage, although embryo survival was affected (Pérez-Cerezales et al., 2010). The risk evaluation of the impact of DNA-damaged germ cells in the reproduction is particularly relevant in animals with external fertilization/embryo development (Pérez-Cerezales et al., 2010), like fish, since both gametes and embryos can be directly exposed to waterborne genotoxicants. This approach can represent an additional contribution to predict the impact of DNA damage on recruitment rate, progeny fitness, and thereby, on the population dynamics. A recent multi-generation study with zebrafish (*D. rerio*) involving a chronic exposure to MMS demonstrated impairments in survival, growth, reproductive capacities and DNA integrity (Faßbender and Braunbeck, 2013). Furthermore, due to the transfer of mutations and inherited DNA damage to the next generation, the offspring was subject to elevated teratogenicity and mortality, pointing out a causal relationship between genotoxicity and the decline of wild populations (Faßbender and Braunbeck, 2013).

Bivalves and Other Molluscs

It must be noted that there are many reports showing reduced genotoxic effects of organic toxicants to molluscs through studies *ex situ* (Parolini and Binelli, 2012; Martins et al., 2013), which, nonetheless, does not relate with technical constraints of the Comet Assay (at least the standard protocols for the alkaline assay are proven to be perfectly effective) but rather on the mechanisms underneath the bioactivation of organic toxicants by multi–function oxidases that, in vertebrates, are responsible for the production of ROS and genotoxic metabolites (Peters et al., 2002). Nevertheless, studies *in situ* with bivalves, at least, often yield good agreement between Comet Assay data and background levels of mixed toxicants, especially organic (Pereira et al., 2011; Martins et al., 2012; Michel et al., 2013). Still, some authors noted the influence of environmental confounding factors, especially, season–related, highlighting increased oxidative stress and DNA strand breaks during warmer months (Almeida et al., 2011; Michel et al., 2013).

The enzyme–modified Comet Assay to detect oxidative DNA damage is just starting to be applied to molluscs, in an attempt to understand the mechanisms underlying DNA damage in these organisms, a subject that still remains largely unknown. It is the case, for instance, of the work by Dallas et al. (2013), who failed to detect Ni–driven Fpg–sensitive (oxidative) DNA damage in the haemocytes of tested mussels, which contradicts *in vitro* studies with humans cells (refer to Cavallo et al., 2003). In another example, Michel and Vincent-Hubert (2012) disclosed that hOGG–1 is more effective in the detection of oxidative damage than alkylated sites (even compared to Fpg) in *D. polymorpha*

gill cells exposed *in vitro* and *in vivo* to a known genotoxicant such as B[α]P. These apparent contradictions showed just how much little is known about the causes and mechanisms of DNA damage and repair in molluscs. In fact, Comet Assay data often yields contradictory or non-linear relations when contrasted to bioaccumulation of genotoxicants and biomarkers related to oxidative stress (such as lipid peroxidation or the activity of antioxidant enzymes), depending on substance, species, and conditions of assessment (e.g., Noventa et al., 2011; Martins et al., 2013). This, again, calls for the need to break way toward the understanding of the fundamental mechanisms underlying genotoxicity in molluscs and their differences to vertebrates, for which most genotoxicity assessment approaches have been devised.

Terrestrial Organisms

E. fetida is extensively used as a compost worm because of its potential to degrade wastes, and has been reared in farms and laboratories for decades. Its continuous exposure to toxic compounds, especially those deriving from agricultural practice, may have been an evolutionary factor for the species. The selective appearance of specific metabolic ways for the detoxification of certain compounds may also result in the activation of other genotoxicants, as has been shown in other species (*Mus musculus* compared with *Apodemus silvatycus*, Acosta et al., 2004). On the other hand, and by a similar reasoning, worms which are native of polluted areas may have developed resistance to those compounds present in their environment.

Discussion and Future Prespectives

The Comet Assay presents several significant advantages over other commonly used assays for genotoxicity studies. Its applicability to both eukaryotic and prokaryotic organism and its use in almost any cell type makes this assay a test very verifiable, reliability, relatively rapidly in data collection and realistic correlation are characteristics also provided by this technique. However, one of the virtues of this assay is unquestionably its cost-effectiveness, compared to many other techniques.

The discussion about the importance of inter-specific differences in sensibility, and on the meaningfulness of using substitute instead of native or target species, is long-lived and still alive, and concerns the core of the toxicological thinking. Indeed, extrapolation is the Achilles heel of toxicology, hence the particular attention given to protocol enhancement and standardization, albeit the need to reason that each case study and each organism need their own set of technical specifications and interpretation requirements, especially considering non-model and moreover, native, species.

There is a wide variety of internal procedures of laboratories where the Comet assay is carried out. As underlined in a previous review article (Frenzilli et al., 2009), the development of suitable guidelines for standardizing Comet Assay protocols is imperative to achieve a harmonization and inter-laboratory calibration. This is also a critical issue to the generalized recognition of Comet Assay as environmental monitoring tool and to allow its integration in regulatory genotoxicological studies. It should be required to the scientist community and to the regulatory agencies to make a meta-analysis or a simple comparison of results obtained from the literature.

Although the Comet Assay has been applied in studies of amphibians, for instance, since the late 1990s, a standardized method to perform the assay and to measure and report this effect does not exist. This represents a disadvantage that limits the comparison with other studies. Despite that, the use of Comet Assay in these organisms is increasing, although it is still limited to the detection of DNA damage. This shows that there is a great potential for development and application of this technique to ecotoxicological studies and environmental risk assessments using amphibians as bioindicator species. The elucidation of the type of DNA damage that is generated and the accurate monitoring of DNA repair through lesion-specific enzymes during the Comet Assay protocol, will add value to this assay in future ecotoxicological studies for exposure assessment and effects on these organisms. Additionally, it could also help to determine the potential causes of their decline in specific environments.

Despite the evidence here highlighted toward a functional association between genotoxicity measured at individual level and a negative impact at population level, so far, DNA damage detected by Comet Assay in fish (as well as in other animal models) has failed to garner sufficient recognition to be incorporated into national and international risk assessment protocols, even though the comparison between this and other potential biomarkers as already showed higher efficiency in the distinction between impacted and reference sites (Costa et al., 2012b). The unequivocal and convincing (mainly for public regulatory agencies) demonstration of its ecological relevance is probably the greatest challenge to Comet Assay on the next decade (goal extensible to majority of biomarkers currently adopted in environmental toxicology).

Another of the many technical constraints that need to be circumvented before the Comet Assay can be efficiently and profusely applied to a wider range of organisms relate to the collection and nature of samples *per se*. For instance, one of the major problems in ecotoxicity terrestrial testing is the high amount of product needed to perform the Comet Assay test. In the case of earthworms, a possible method to reduce the amount of test material required is to inject the test solution directly in the coelomic cavity of the earthworms; this is how was conducted the recently reported Comet Assay study of functionalized-quantum dots (QDs) and cadmium chloride on *Hediste diversicolor* and *E. fetida* coelomocytes. Results demonstrated that functionalized-QDs (QDNs) and cadmium chloride induced DNA damages through different mechanisms that depended on the nano- or ionic nature of Cd (Saez et al., 2014). Spiked soil should be allowed to stabilize for a sufficient period before starting the exposition test to performing the Comet Assay. This time, necessary to reach a status of equilibrium similar to that established in natural conditions, is probably too short in most studies. On the other hand, the nature and circumstances of soil in the real polluted areas may dramatically affect the bioavailability of xenobiotics. Time and exposure to the action

of weather tends to have a homeostatic effect, decreasing the access of toxicants to the internal medium of living organisms. This partially accounts for the surprisingly mild effects frequently observed in areas which chemical analysis have shown to be heavily polluted (Alexander and Alexander, 2000; Borràs and Nadal, 2004; Vasseur and Bonnard, 2014). As a consequence, experiments with spiked soil could tend to show a higher degree of toxic effects, being more sensitive but also, possibly, less realistic. Still regarding this issue, a way to avoid the large amounts of sample needed in a conventional growth test in soil consists in treating only the exposed root tips. For example, *Allium cepa* root tips were treated with TiO_2 nanoparticles dispersions at four different concentrations (12.5, 25, 50, 100 mg/mL). The bio-uptake of TiO_2 in particulate form was the key cause of ROS generation, which in turn was probably the cause of the DNA aberrations and genotoxicity (Ghosh et al., 2010; Panda et al., 2011; Pakrashi et al., 2014).

Overall, these few examples clearly illustrate that the application of the Comet Assay in ecogenotoxicity assessment remains as purposeful as challenging. The swift integration of novel methodological improvements to the protocol with this field of research, such as DNA repair enzyme modifications, shows that ecotoxicologists are constantly improving approaches and protocols. Furthermore, it must be noticed, as hereby demonstrated, that ecotoxicology is probably one of the most diversified and complex field of research where genotoxicity assessment is surveyed as routine. As such, one may expect another further decades of successful, although constantly improving, application of this versatile protocol.

Internet Resources

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An integrative assessment to determine the genotoxic hazard of estuarine sediments: combining cell and whole-organism responses

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The application of the Comet assay in environmental monitoring remains challenging in face of the complexity of environmental stressors, e.g., when dealing with estuarine sediments, that hampers the drawing of cause-effect relationships. Although the *in vitro* Comet assay may circumvent confounding factors, its application in environmental risk assessment (ERA) still needs validation. As such, the present work aims at integrating genotoxicity and oxidative DNA damage induced by sediment-bound toxicants in HepG2 cells with oxidative stress-related effects observed in three species collected from an impacted estuary. Distinct patterns were observed in cells exposed to crude mixtures of sediment contaminants from the urban/industrial area comparatively to the ones from the rural/riverine area of the estuary, with respect to oxidative DNA damage and oxidative DNA damage. The extracts obtained with the most polar solvent and the crude extracts caused the most significant oxidative DNA damage in HepG2 cells, as measured by the formamidopyrimidine-DNA glycosylase (FPG)-modified Comet assay. This observation suggests that metals and unknown toxicants more hydrophilic than polycyclic aromatic hydrocarbons may be important causative agents, especially in samples from the rural part of the estuary, where oxidative DNA damage was the most significant. Clams, sole, and cuttlefish responded differentially to environmental agents triggering oxidative stress, albeit yielding results accordant with the oxidative DNA damage observed in HepG2 cells. Overall, the integration of *in vivo* biomarker responses and Comet assay data in HepG2 cells yielded a comparable pattern, indicating that the *in vitro* FPG-modified Comet assay may be an effective and complementary line-of-evidence in ERA even in particularly challenging, natural, scenarios such as estuarine environments.

Keywords: Comet assay, environmental risk assessment, sediment contamination, oxidative stress, HepG2 cells

INTRODUCTION

Ever since the original publication of the protocol by Singh et al. (1988), the alkaline Comet assay rapidly developed into one of the most prolific tools for those performing research on environmental genotoxicity. Indeed, this paramount technical achievement quickly became one of the most important tools to assess the hazards of genotoxicants in the environment, with emphasis on the aquatic milieu (see Mitchelmore and Chipman, 1998). Within these ecosystems, sediments have been targeted in environmental risk assessment (ERA) studies due to their ability to trap, store, and (depending on disruption of their steady-state) release contaminants back to the biota. The range of these substances includes genotoxicants, from metals to dioxins and polycyclic aromatic hydrocarbons (PAHs), the latter being highly hydrophobic mutagens and holding high affinity to organic matter and fine fraction (see Chen and White, 2004, for a review).

It is becoming increasingly common to employ *in vitro* approaches with fish cell lines exposed to aquatic sediment extracts to determine the genotoxic potential of bioavailable pollutants (for instance, Kosmehl et al., 2008; Yang et al., 2010; Šrut et al., 2011). In contrast, similar work with human cell lines is less common. The relatively simple logistics of *in vitro* assays renders their combination with the Comet assay appealing for the determination of the genotoxic effects of pollutants in sediment and water samples. In particular, the human hepatoma HepG2 cell line has long been regarded as metabolically competent to determine genotoxic effects of chemical substances, with proven sensitivity for the detection of such effects through the Comet assay (Uhl et al., 1999). Still, regardless of being logistics-friendly and able to reduce much of the confounding factors that often hinder the interpretation of results when testing or sampling *in situ* aquatic organisms, it is clear that the results obtained *in vitro* need to be compared with

other lines-of-evidence in order to obtain practical validation for the purpose of ERA.

The analysis of biomarker responses related to oxidative stress is deemed to be indicative of reactive oxygen species (ROS) produced directly or indirectly as a consequence of exposure to xenobiotics. As such, oxidative stress biomarkers allow a pertinent approach to evaluate sub-individual effects of toxicological challenge and therefore enable an overall assessment of the effects of environmental contaminants or their mixtures (see, for instance, van der Oost et al., 2003; Picado et al., 2007). Oxidative-stress related biomarkers in vertebrate or invertebrates have been proposed for ERA under a multiplicity of scenarios, whether concerning specific substances, classes of substances or particularly challenging mixtures as aquatic sediments (e.g., van der Oost et al., 2003; Scholz et al., 2008; Bonnineau et al., 2012; Chapman et al., 2013). Nevertheless, biomarkers such as lipid peroxidation and the activity of anti-oxidant enzymes may be modulated by many confounding factors and by distinct types of both organic and inorganic toxicants, rendering difficult the determination of cause–effect relationships. This may be particularly critical when addressing complex contaminant matrices such as aquatic sediments (see Chapman et al., 2013, for a recent review). Still, as for other biomarker responses, measuring oxidative damage and defenses in wild organisms has long become an important component of ERA. Oxidative radicals are responsible for the dysregulation of many cellular functions and for damage to molecules, including DNA (reviewed by Cadet et al., 2010). As a consequence, the recent developments in Comet assay protocols combining enzymes involved in the repair of oxidative DNA damage are breaking ground to link toxicant-induced oxidative stress and DNA damage (see Collins, 2009, 2014, and references therein).

Studies attempting to integrate DNA damage retrieved from the *in vitro* Comet assay and biomarker responses of field-collected animals are lacking, which constitutes a gap within the validation of cell-based assays in ERA, despite the acknowledged importance of genotoxicity as a line-of-evidence (LOE). The present study aims essentially at comparing the performance, as ecotoxicological indicators, of the formamidopyrimidine-DNA glycosylase (FPG)-modified Comet assay in HpG2 cells exposed to sediment-bound contaminants with that of common oxidative stress-related biomarkers determined in three distinct organisms collected from an impacted estuarine area. Ultimately, it was intended to contribute for the validation of the data produced by the *in vitro* Comet assay as a LOE in ERA strategies. For this purpose, the present study integrates and re-interprets the findings from recent research on the Sado Estuary (SW Portugal), taken as the case study, and presents for the first time data from the *in vitro* analysis of sediment extract fractioning.

MATERIALS AND METHODS

STUDY AREA AND SAMPLE COLLECTION

The Sado estuary, located in SW Portugal, consists of a large basin of high ecological and socio-economical importance. The estuary is very heterogeneous, with respect to its biogeography and anthropogenic use. The basin includes the city of Setúbal, with its harbor and heavy-industry belt, located in the northern area (Sado 1). On its turn, the southern region (Sado 2), where the mouth of the river Sado is situated, is essentially agricultural (**Figure 1**). Part of the estuary is classified as a natural reserve and, besides industry and shipping, the estuary is also very important for tourism, fisheries, and aquaculture. The river itself transports to the estuary fertilizers, pesticides from run-offs of the agriculture grounds upstream

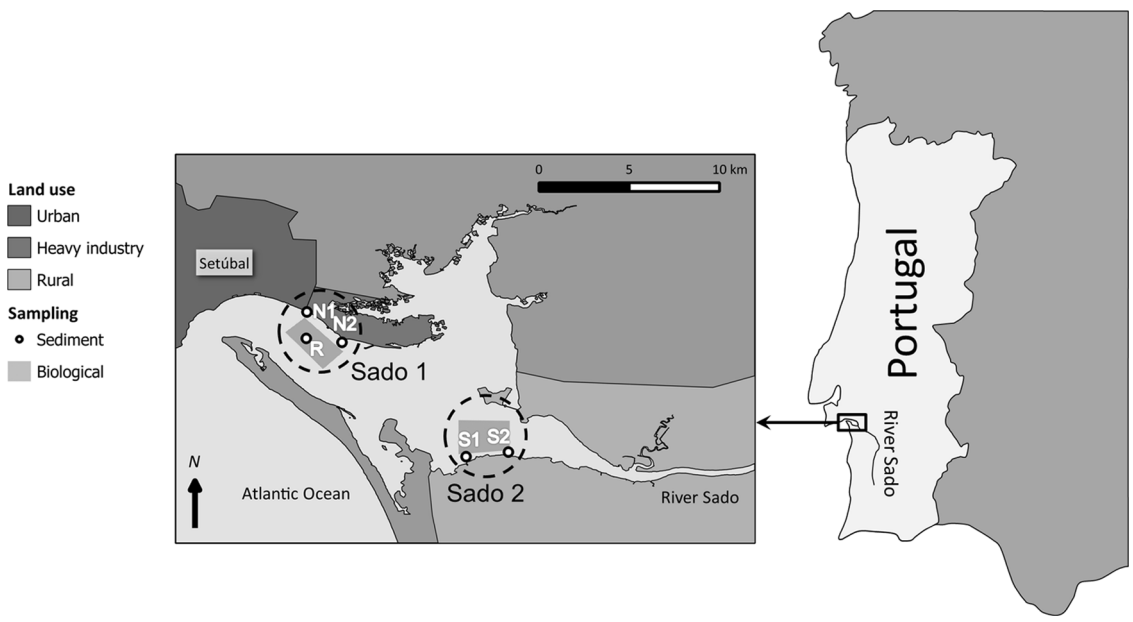

FIGURE 1 | Map of the river Sado Estuary, Portugal, highlighting the two study areas: Sado 1 (north) and Sado 2 (south). Sediment collection sites and organism fishing grounds for each area are also indicated. Refer to the legend for specifics.

and metals from pyrite mining areas. The estuary has been judged to be globally moderately impacted by pollutants albeit ecotoxicologically diversified (refer to Caeiro et al., 2009, Costa et al., 2012, and references therein). Altogether, the multiple human activities result in diverse sources of contamination (most of which diffuse) and dictate the need to develop effective environmental managing and land use plans that include monitoring the presence, fate and effects of potential pollutants.

Sediment samples were collected from five different sites within the Sado estuary between spring 2007 and spring 2010. Sites N1 and N2 (Sado 1) are located off Setúbal's harbor and industrial belt, respectively. Sites S1 and S2 (Sado 2), in the southern part of the estuary are located near an agricultural region with direct influence from the River Sado (**Figure 1**). The reference sediment (R) was collected from a sandy shellfish bed with high oceanic influence, from where clams were collected (see Carreira et al., 2013). Metallic/metalloid and organic toxicants (PAHs and organochlorines) were analyzed in sediments by means of inductively-coupled plasma mass spectrometry (ICP-MS) and gas chromatography–mass spectrometry techniques, respectively, with the results being validated through the analyses of certified reference materials (refer to Costa et al., 2011 and Carreira et al., 2013, for procedural details). Clams (*Ruditapes decussatus*) were collected from sites R and S1 upon sediment collection. Fish (*Solea senegalensis*), and cuttlefish (*Sepia officinalis*) were collected from acknowledged fishing grounds in Sado 1 and Sado 2 (**Figure 1**). Fish and cuttlefish biomarkers were contrasted to data of animals collected outside the estuary, within the same geographical region. However, sediment analyses (for pollutants, grain size, redox potential, and organic matter) from this external area yielded similar results to that of sediment R, which was found to be essentially devoid of any significant contamination, in spite of its proximity to sites N1 and N2. For such reason, oxidative stress biomarker data from fish and cuttlefish were geographically allocated to site R, for computational purposes. In order to congregate sediment toxicant levels into more manageable indices, these data were used to estimate sediment quality guideline quotients (SQG-Qs) for each class of contaminants and for total contamination, according to Long and MacDonald (1998), following contrasting to the probable effects level (PEL) guidelines for marine pollutants, available for most analyzed substances (MacDonald et al., 1996). The SQG-Q scores provide a measure of risk, allowing sediments to be classified as unimpacted if SQG-Q < 0.1; moderately impacted if $0.1 <$ SQG-Q < 1 and highly impacted if SQG-Q > 1 (MacDonald et al., 2004). **Table 1** summarizes the main sediment contamination data and respective SQG-Qs. Sediment data were retrieved from Costa et al. (2011) and Carreira et al. (2013).

SEDIMENT EXTRACTS

Sediment contaminant extraction follows the protocol of Šrut et al. (2011), with few modifications, as described in detail by Pinto et al. (2014b). In summary, pulverized dry sediment samples were subjected to mechanical extraction with a series of organic solvents of increasing polarity. Fraction 1 (the crude extract) was obtained with a dichloromethane (DCM):methanol (2:1) mixture to attempt extraction of the bulk toxicants; fraction 2 with *n*-hexane (apolar); fraction 3 with DCM, and fraction 4 with methanol (the most polar solvent). The solvents were afterward evaporated at 45°C and the extracts reconstituted in dimethyl sulfoxide (DMSO). The concentrations of the extracts were estimated as mg sediment equivalent (SEQ) per mL of cell culture medium.

IN VITRO ASSAYS

The human hepatocellular carcinoma cell line (HepG2) was obtained from the American Type Culture Collection (ATCC ref. HB-8065) and cultured as described in Pinto et al. (2014a,b). Cytotoxicity was measured through the neutral red (NR) assay, performed in triplicate for each experimental condition, as previously described (Pinto et al., 2014b). Briefly, after a 48 h exposure period to sediment extracts (from 5 up to 200 mg SEQ/mL), HepG2 cells were incubated with NR (3 h), which was afterward recovered and measured spectrophotometrically (540 nm). The relative cell viability, expressed as the percentage of viable cells, was estimated by the ratio between the mean absorbance of treated and control cells, assuming the mean absorbance of the negative control to represent 100% viable cells. The level of DNA damage and oxidative DNA damage was evaluated by the Comet assay and FPG-modified Comet assay, respectively, the latter to convert oxidized purines into single-strand breaks (Collins, 2009). The experiment was performed in triplicate. In brief: following a 48 h exposure period to each sediments extract, HepG2 cells were washed, detached, embedded in low-melting point agarose (1% m/v) and spread onto duplicate gels per replicate. Cells were then lysed (for at least 1 h) before nucleoid treatment with FPG or buffer only (30 min, 37°C). DNA was allowed to unwind (40 min) before electrophoresis (0.7 V/cm, 30 min). After staining with ethidium bromide, one hundred randomly selected nucleoids were analyzed per experimental condition. The mean percentage of DNA in tail was taken as the final endpoint for being regarded as one of the most consistent Comet metrics (Duez et al., 2003).

BIOMARKER APPROACH

The multiple oxidative stress-related biomarker responses in wild organisms were retrieved from Carreira et al. (2013), Gonçalves et al. (2013), and Rodrigo et al. (2013), for clam, sole, and cuttlefish, respectively. The molluscan digestive gland and fish liver were chosen as target organs for being analog organs and due to their role in the storage and detoxification of xenobiotics. The oxidative stress-related biomarkers investigated in the present study were lipid peroxidation and catalase (CAT) activity in clams; lipid peroxidation, catalase activity, and glutathione *S*-transferase (GST) activity in fish; lipid peroxidation, GST activity, total glutathione (GSHt), and reduced/oxidized glutathione ratio (GSH/GSSG) in cuttlefish. Details of the procedures can be found in Carreira et al. (2013), Gonçalves et al. (2013), and Rodrigo et al. (2013). Briefly: GSHt was determined as through the enzymatic recycling method, using a commercial kit (Sigma–Aldrich), following manufacturer instructions. The GSH/GSSG ratio was estimated following derivatization of subsamples with 2-vinylpyridine (Sigma–Aldrich), in order to obtain the GSSG concentration. The ratio was determined as GSH/(GSSG/2). The activity of GST was determined

Table 1 | Sediment contamination data and respective sediment quality guideline quotients (SQG-Qs) per sediment sample.

	Area	Sado 1		Sado 2		
	Site	R*	N_1**	N_2*	S_1*	S_2*
Metal (μg/g)						
Metalloid	As	0.34 ± 0.26	23.98 ± 0.48	19.7 ± 5.21	26.44 ± 2.68	25.02 ± 8.84
	Se	1.84 ± 0.84	1.21 ± 0.02	1.92 ± 1.45	0.59 ± 0.21	0.72 ± 0.08
Metal	Cr	2.36 ± 0.36	80.73 ± 1.61	77.67 ± 4.57	62.22 ± 4.45	87.61 ± 2.97
	Ni	4.10 ± 1.66	33.30 ± 0.67	16.67 ± 1.1	17.15 ± 1.21	22.79 ± 9.47
	Cu	4.51 ± 1.05	172.72 ± 3.45	178.64 ± 7.01	74.15 ± 13.16	92.3 ± 5.63
	Zn	13.10 ± 1.51	364.83 ± 7.30	327.51 ± 1.16	269.79 ± 7.81	385.11 ± 35.69
	Cd	0.03 ± 0.02	0.26 ± 0.01	0.27 ± 0.03	0.33 ± 0.13	0.43 ± 0.19
	Pb	3.50 ± 0.48	55.19 ± 1.10	56.45 ± 3.1	25.3 ± 0.91	32.7 ± 1.21
Organic (ng/g)						
	tPAH	19.60 ± 3.33	1 365.20 ± 232.08	1.076.98 ± 183.09	215.03 ± 36.55	82.47 ± 14.02
	tDDT	0.02 ± 0.00	0.37 ± 0.06	1.22 ± 0.21	0.21 ± 0.04	0.13 ± 0.02
	tPCB	0.05 ± 0.01	7.91 ± 1.34	5.37 ± 0.91	0.26 ± 0.04	0.27 ± 0.05
SQG-Q	$SQG\text{-}Q_{metal}$	0.04	0.79	0.68	0.62	0.49
	$SQG\text{-}Q_{organic}$	0.00	0.09	0.06	0.01	0.00
	$SQG\text{-}Q_{total}$	0.02	0.33	0.37	0.31	0.25
Impact status		Unimpacted	Moderate	Moderate	Moderate	Moderate

**data from Carreira et al. (2013); **data from Costa et al. (2011).*

spectrophotometrically using commercial kit (Sigma–Aldrich), following the instructions from the manufacturer, by measuring the increase in absorbance at 340 nm during 5 min, using chloro-2,4-dinitrobenzene (CDNB) as substrate. Lipid peroxides were determined through the thiobarbituric acid-reactive species (TBARS) assay developed by Uchiyama and Mihara (1978) and adapted by Costa et al. (2011). Samples were homogenized in cold phosphate-buffered saline, PBS (pH 7.4, with 0.7% NaCl) and the supernatant was deproteinated with trichloroacetic acid, after which thiobarbituric acid was added and the samples incubated for 10 min in boiling water. The absorbance of reddish pigment was measured at 530 nm and quantified through a calibration curve using malondialdehyde bis(dimethylacetal), from Merck, as standard. CAT activity was measured spectrophotometrically (at 240 nm during 6–8 min at 30 s intervals) according to method of Clairborne (1985), being estimated as units (U) per mg protein. All biomarker responses were normalized to sample total protein, determined through the method of Bradford (1976). The biomarker data are summarized in **Table 2**.

EC_{50} ESTIMATION

The half-maximal effective concentration (EC_{50}) for cytotoxicity and genotoxicity was estimated for crude and fractionated extracts to allow the comparison of their relative cytotoxic and genotoxic potencies (see Seitz et al., 2008). Genotoxicity EC_{50} (with and without FPG treatment) was estimated by considering the highest measured %DNA in tail throughout the experiments as the maximal effect, since the %DNA in tail should not reach 100%. The EC_{50} values were estimated from normalized data through log-logistic regression and were computed using Stat4Tox 1.0 (Joint Research Centre of the European Commission), built for the R platform (Ihaka and Gentleman, 1996), version 2.10. Estimates are provided as mg SEQ/mL ± 95% confidence intervals.

INTEGRATED BIOMARKER RESPONSE

The integrated biomarker response (IBR) indice was computed to integrate oxidative-stress biomarker responses determined in cuttlefish digestive gland (GST, GSHt GSH/GSSG, LPO), flatfish liver (CAT, GST, LPO), and clam digestive gland (CAT, LPO), according to the method described by Beliaeff and Burgeot (2002). Accordingly, the IBR is based on the partial score (*S*) estimates for each biomarker and organism. The scores were used to calculate the area (*A*) connecting consecutive coordinates (data points) in star plots. The IBR for each area (Sado 1, Sado 2, and the reference scenario) and *S* for each species were then calculated through the sum of the respective *A* values. See Rodrigo et al. (2013) for further details. The modifications suggested for IBR calculations, specifically the transformation to IBR/number of biomarkers (e.g., Broeg and Lehtonen, 2006), were not applied since for every area the same organisms and biomarkers were analyzed.

STATISTICS AND INTEGRATION OF DATA

Data were mapped through a geographical information system (GIS) approach using QGis 2.0 and the digital map for mainland coastal waters (EPSG:4326 – WGS 84 coordinate system) made available by the Hydrographic Institute of the Portuguese

Table 2 | Mean biomarker data (±SD) analyzed in the present work, for each species collected from the three study areas: Sado 1 (north); Sado 2 (south), and Reference.

		CAT (U/mg protein)	GST (nmol/min/mg protein)	GSHt (nmol/mg protein)	GSH/GSSG	LPO (nmol/mg protein)
Area						
Sado 1						
	Clam	24.75 ± 22.37				0.002 ± 0.001
	Fish	24.54 ± 21.94	0.12 ± 0.11			1.76 ± 1.05
	Cuttlefish		0.005 ± 0.002	0.11 ± 0.13	2.08 ± 2.39	0.69 ± 0.38
Sado 2						
	Clam	33.37 ± 27.84				0.003 ± 0.002
	Fish	46.91 ± 26.23	0.31 ± 0.14			1.26 ± 0.72
	Cuttlefish		0.003 ± 0.001	0.04 ± 0.08	2.22 ± 2.02	0.57 ± 0.32
Reference						
	Clam	18.70 ± 9.39				0.001 ± 0.000
	Fish	25.34 ± 20.64	0.21 ± 0.09			1.05 ± 0.52
	Cuttlefish		0.002 ± 0.001	0.04 ± 0.03	2.82 ± 1.73	0.23 ± 0.09

Data from clam (Ruditapes decussatus), sole (Solea senegalensis), and cuttlefish (Sepia officinalis) were retrieved from Carreira et al. (2013), Gonçalves et al. (2013), and Rodrigo et al. (2013), respectively.

Navy (http://www.hidrografico.pt). In order to obtain a general overview of the sediments' contamination status, SQG-Q values for total contamination, metals, and organic toxicants were used for the analysis. The approach included also the EC_{50} estimates obtained from the Comet assay data (with and without FPG treatment) plus the global IBR for each area (combining all species and biomarkers). Interpolation of data points to raster layers was achieved through the inverse distance weight (IDW) algorithm from minimum–maximum normalized values.

Cluster analysis was done using Cluster 3.0, integrating SQG-Qs, EC_{50} estimates from the Comet assay and IBR values. Dendrograms and heatmaps were plotted using Java TreeView 1.1.6. Additional correlation statistics (Spearman's *R*) and the Kruskall–Wallis Median Test adaptation for multiple comparisons (following recommendations by Duez et al., 2003) were computed with Statistica 8.0 (Statsoft).

Table 3 | Cytotoxicity EC_{50} estimates for HepG2 cells exposed to each extract fraction for all surveyed sediment samples (in mg SEQ/mL).

	Extract fraction			
Site	**1**	**2**	**3**	**4**
R	–	–	–	–
N1	39.8 (34.3–45.2)	n.a.	n.a.	n.a.
N2	88.7 (82.1–95.4)	–	–	265.3 (158.5–372.0)
S1	180.0 (162.7–197.3)	–	–	–
S2	223.5 (152.5–294.6)	–	–	160.9 (70.1–251.8)

[–], not computable (effect too low); n.a., data not available; fraction 1, dichloromethane:methanol (crude extract); fraction 2, n-hexane; fraction 3, dichloromethane; fraction 4, methanol; ranges indicate the lower and upper 95% confidence limits.

RESULTS

The cytotoxicity of the different extracts, as evaluated by EC_{50} estimates (**Table 3**), was highly variable. All extracts from the reference sediment (R) failed to yield significant cytotoxicity at the tested concentrations. Similar results were obtained for fractions 2 and 3 of any sediment. The lowest EC_{50} estimates, indicating higher cytotoxic potency, were obtained for fraction 1 (crude extract) of samples N1 and N2 (Sado 1 area). The cytotoxicity data were used to select the dose-range for genotoxicity testing, in order to avoid interference from cytotoxic events causing DNA strand breakage.

Examples of Comet nucleoids from exposed HepG2 cells are given in **Figure 2**. Non-oxidative strand breakage (**Figure 3A**) tended to increase with extract concentration, especially following exposure to extract fractions 1 and 4. Overall, DNA strand breakage was accentuated by the FPG-linked Comet assay (**Figure 3B**). The increase in total DNA damage in FPG-treated HepG2 cells was more pronounced following exposure to extracts S1 and S2 (especially fractions 1 and 4), attaining approximately 30% of DNA in the nucleoids' tail. Conversely, no sizable effects were observed in cells exposed to any of the extracts from sediment R.

The EC_{50} estimates for DNA strand breakage revealed distinct trends between estuarine areas, sediment samples, and oxidative/non-oxidative damage (**Table 4**). Cells exposed to the crude extracts of Sado 1 sediment samples N1 and N2 yielded the lowest EC_{50} estimates for both FPG-treated and non-treated samples (meaning higher DNA damage at similar SEQ). In general, the FPG-modified Comet assay, which includes oxidative damage, resulted in decreased EC_{50} estimates. Furthermore, comparing data from the FPG-modified Comet assay to the conventional assay evidenced that the highest increase in oxidative DNA strand breakage occurred following exposure to sediment extract S1, fraction 1 (resulting in EC_{50} reduction by almost fourfold), and S2, fraction 1 (DCM:methanol) as well, for which no computable

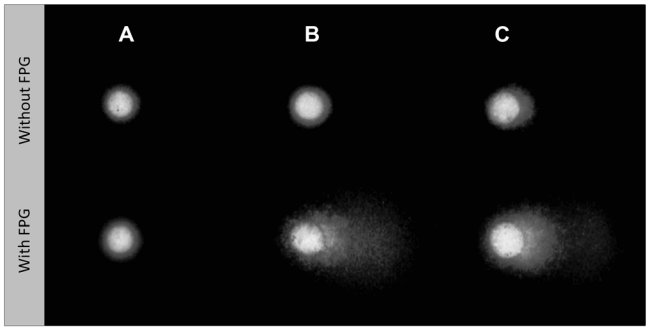

FIGURE 2 | Representative HepG2 Comet nucleoids treated without or with formamidopyrimidine-DNA glycosylase (FPG), to reveal oxidative damage to DNA. (A) Negative control (dimethyl sulfoxide, DMSO only). **(B)** Cells exposed to the crude extract from sediment S1 [100 mg sediment equivalent (SEQ)/mL]. **(C)** Cells exposed to the crude extract from sediment S2 (200 mg SEQ/mL).

EC_{50} could even be retrieved from the conventional Comet assay. Overall, fractions 2 (*n*-hexane) and 3 (DCM) failed to produce estimates due to low induction of genotoxic effects. No EC_{50} values could be estimated from data of cells exposed to any of the fractions from the reference sediment (R). No correlations were found between cytotoxicity EC_{50} and DNA strand breakage EC_{50} estimates, with or without FPG-treatment (Spearman's R, $p > 0.05$).

Clam, fish, and cuttlefish yielded distinct patterns of oxidative biochemical damage (measured through lipid peroxidation) and responses to oxidative stress (see **Table 2**). In accordance, distinct IBR scores were obtained from each surveyed species. However, the aggregated results indicate a similar trend to increase oxidative stress responses and effects in animals collected from the impacted sites Sado 1 (IBR = 2.10) and Sado 2 (IBR = 2.72), compared to the reference scenario (IBR = 0.01), when combining all three species (**Figure 4A**). Clams, for which lipid peroxidation and CAT activity were surveyed, yielded higher IBR scores for Sado 2 (**Figure 4B**), similarly to fish (**Figure 4C**), for which GST was added. Conversely, cuttlefish, for which lipid peroxidation, GST activity, GSHt, and reduced/oxidized glutathione ratio were surveyed, yielded higher IBR for Sado 1 (**Figure 4D**).

Spatial distribution of data for sediment contamination plus Comet assay and IBR results are presented in **Figure 5**. The distribution of sediments contaminants was found to be very heterogeneous within the estuary, marking a distinction between Sado 1 (urban and industrial) and Sado 2 (rural and riverine) areas (**Figures 4A–C**), with the reference site evidencing a clear distinction from its immediate surroundings. The distinction between Sado 1 and Sado 2 is more obvious for organic contaminants, of which PAHs (**Figure 5C**) are the most representative (see **Table 1** also). These contaminants were best represented in Sado 1 sediments N1 and N2, in line with the findings retrieved from the conventional Comet assay (**Figure 5D**). Oxidative DNA strand breakage increased most notoriously in HepG2 cells exposed to sediments from Sado 2 (**Figure 5E**). Accordingly, animals from Sado 2 yielded comparatively the highest combined IBR value for oxidative stress-related biomarkers (**Figure 5F**). In agreement with the spatial distribution of data, cluster analyses combining sediment and biological data grouped sites N1 and N2 within the same cluster, both belonging to Sado 1 whereas sites S1 and S2 (Sado 2) constituted a clearly distinct group. Still, the Reference site (R) exhibited a closer relation to Sado 2 than to Sado 1 sites (**Figure 6**). Oxidative DNA damage caused by exposure to fraction 1 was best correlated to IBR and, together with SQG-Qs for metals and total toxicants, formed a distinct cluster from the one (cluster 2) comprising SQG-Qs for organic toxicants, non-oxidative DNA damage, and oxidative DNA damage resulting from exposure to the extract fractions 4 (methanol).

DISCUSSION

The present work showed that estuaries, even if regarded as moderately impacted, may be highly heterogeneous with respect to the distribution of pollutants which, consequently, is translated into a complex pattern of biological effects and responses to toxicants. Oxidative DNA damage was found to be associated to IBR estimates (**Figure 6**), for oxidative stress biomarkers analyzed in local species (combining clams, fish, and cuttlefish). This indicates a relationship, as potential lines-of-evidence, between two distinct sets of oxidative effects, i.e., biochemical and genetic, determined in wild animals, and HepG2 cells, respectively.

Overall, the results indicate that oxidative effects endured by wild organisms and HepG2 cells are better associated either to total contamination or to metals (the best represented toxicants), than to well-known genotoxicants like PAHs. It must be emphasized that sediment contamination, with particular respect to organic contaminants (among which PAHs are the best represented), was globally higher in sediments N1 and N2 (i.e., from the industrial area of the estuary). Nonetheless, the increment of DNA strand breakage in FPG-treated cells relatively to the standard assay was higher in HepG2 cells after exposure to extracts from Sado 2 (the rural and riverine area), indicating a higher level of oxidative DNA damage. This observation is accordant with the present IBR results and also as disclosed by the original research with cells with unfractionated extracts (see Pinto et al., 2014a for further details). In fact, under this scope, the analyses with this cell line provided a globally more conclusive distinction between contaminated and reference areas than each species individually, since clams, fish, and cuttlefish yielded different results (**Figure 4**). However, any potential link between oxidative DNA damage in cells and biochemical oxidative stress in wild organisms remains elusive, since organisms hold specific abilities to cope with exposure to toxicants and the oxidative stress hitherto derived.

Past research to determine the effects of sediment contamination in the Sado Estuary based on a multi-biomarker approach in soles exposed *in situ* and *ex situ* revealed that the *in vivo* Comet assay provided one of the most consistent measurements to distinguish contaminated from non-contaminated sites, among a wide battery of biomarkers (Costa et al., 2012). Oppositely, Gonçalves et al. (2013) disclosed that anti-oxidative defenses, namely the activity of CAT and GST were inhibited in fish from Sado 1, where highest lipid peroxidation levels occurred. These findings are thus accordant with reduced IBR scores in animals from Sado 1 (**Figure 4C**). The same authors then hypothesized that one of the

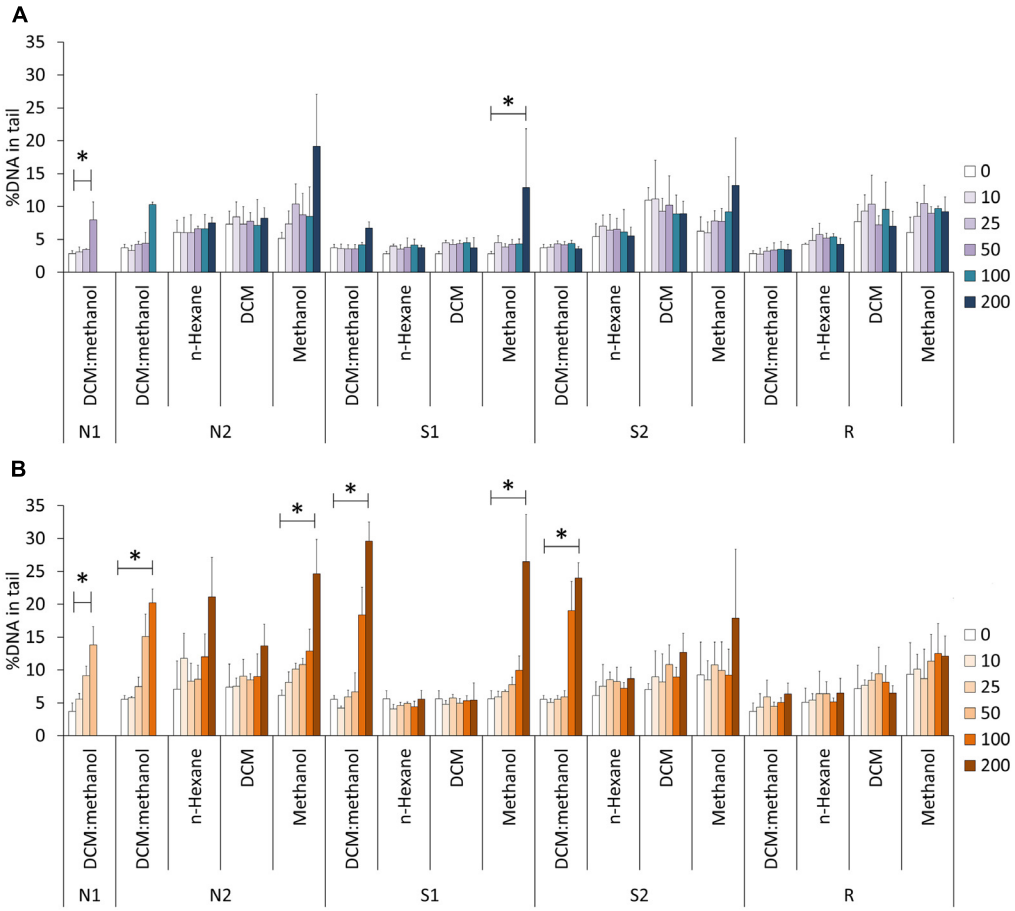

FIGURE 3 | Comet assay results in HepG2 cells exposed to the different sediment extracts at different concentrations (0–200 SEQ mg/mL). **(A)** Experiments without FPG treatment. **(B)** Results from the FPG-linked Comet assay. *Indicates significant differences between multiple concentrations (Kruskall–Wallis Median Test, $p < 0.05$). The results are indicated as mean %DNA in tail ± SD. Dada from DCM:methanol extracts were retrieved from Pinto et al. (2014a). The concentration 0 mg SEQ/mL corresponds to the negative (solvent) control (DMSO only).

factors involved in such inhibition was the complex interaction of toxicants (organic and metallic). Altogether, when comparing the effects on fish and human cells, it may be inferred that oxidative stress occurs indeed as a consequence of exposure to toxicants from Sado 1, whether translated into oxidative DNA lesions or biochemical damage. This information is in agreement with higher levels of contamination by organic compounds, especially PAHs, since metals presented similar values between the two main areas of the estuary (**Table 1**). On the other hand, molluscs provided consistent, albeit opposite, responses that are related to habitat and behavior. Clams (sedentary burrowers) from Sado 1 were collected from the precise same site than sediment R (the "clean" reference sediment); so, not surprisingly the IBR score was lower in comparison to clams collected from Sado 2 (specifically, from site S1). On its turn, cuttlefish (a foraging, territorial, predator) was consistently responsive to background contamination of Sado 1. Yet, these animals are a novelty within the field of research and little is known about its physiological responses to chemical challenge (see Rodrigo et al., 2013, for details).

The current findings are partially accordant with those obtained by Šrut et al. (2011) and Pinto et al. (2014b), who revealed higher strand breakage in a fish and human hepatoma cell line, respectively, exposed to crude extracts (dichloromethane:methanol) of marine sediments, when compared to exposure to fractions obtained with increasingly polar solvents. In fact, the significant correlations between EC_{50} estimates (oxidative and non-oxidative DNA damage) and SQG-Qs for organic and inorganic toxicants indicate that this extraction method was efficient for the bulk of toxicants (**Figure 6**). However, in the present study, fractions 2 (*n*-hexane) and 3 (dichloromethane) yielded only marginal results. Considering that metals are indeed the most significant toxicants determined in Sado sediments from contaminated areas, the results are in line with SQG-Qs (**Table 1**), since exposure to fraction 2 should mean exposure to PAHs and other highly hydrophobic substances. Moreover, it was observed that sediments from Sado 2 (S1 and S2) account primarily for oxidative DNA damage in HepG2 cells, showing that distinct sets of sediment toxicants were retrieved from both Sado areas (**Figure 2**; **Table 3**). Most likely, Sado 2 sediments contain important levels

Table 4 | DNA damage EC_{50} estimates (retrieved from the % of DNA in tail) for HepG2 cells exposed to each extract fraction for all surveyed sediment samples (in mg SEQ/mL) relatively to the maximum observed %DNA in tail throughout the study (≈30%).

	Extract fraction			
Site	**1**	**2**	**3**	**4**
Alkaline Comet				
R	–	–	–	–
N1	82.0 (34.8–129.1)	n.a.	n.a.	n.a.
N2	131.6 (103.6–159.6)	–	–	195.6 (19.1–374.1)
S1	364.5 (238.2–490.7)	–	–	223.9 (168.4–279.4)
S2	–	–	–	–
Alkaline Comet + FPG				
R	–	–	–	–
N1	65.4 (59.6–71.2)	n.a.	n.a.	n.a.
N2	72.6 (53.2–91.9)	175.5 (99.0 – 252.0)	354.6 (86.5–622.7)	127.8 (72.3–183.3)
S1	97.1 (90.2–104.0)	–	–	136.4 (117.4–155.4)
S2	104.1 (73.0–135.2)	–	–	–

[–], not computable (effect too low); n.a., data not available; fraction 1, dichloromethane:methanol (crude extract); fraction 2, n-hexane; fraction 3, dichloromethane; fraction 4, methanol; ranges indicate the lower and upper 95% confidence limits.

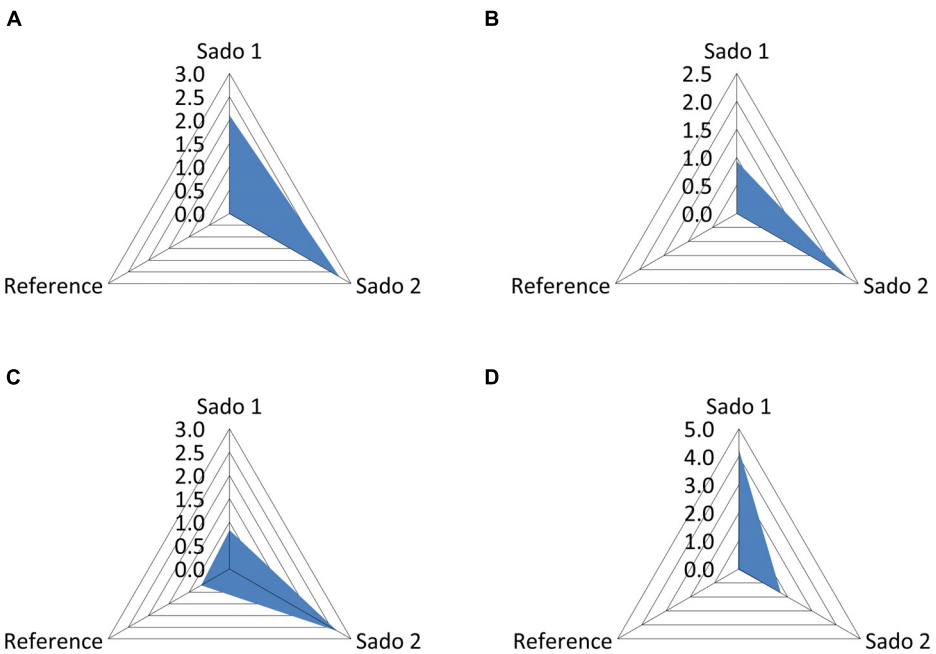

FIGURE 4 | Plots of the integrated biomarker response (IBR) for the three areas, Sado 1 (urban and industrial), Sado 2 (riverine and agricultural) and Reference. (A) Global IBR combining clam, fish (sole), and cuttlefish; IBR scores (*S*) for clam **(B)**; fish **(C)**, and cuttlefish **(D)**.

of more hydrophilic toxicants, such as metals and potentially unsurveyed organic substances, either able to cause oxidative DNA damage or some type of alkylating lesions that might have been converted in strand breaks following FPG treatment (see Collins, 2014).

It must be noted that HepG2 cells have already been found sensitive to metal-induced DNA strand breakage measurable by the standard Comet assay, albeit yielding non-linear cause-effect relationships likely due to adequate deployment of defenses such as metallothioneins (Fatur et al., 2002). These findings have been confirmed through the exposure of HepG2 cells to metals extracted from soils (in aqueous phase), revealing, nevertheless, reduced sensitivity (Vidic et al., 2009). Still, unlike the present study, oxidative DNA damage was not measured in these works.

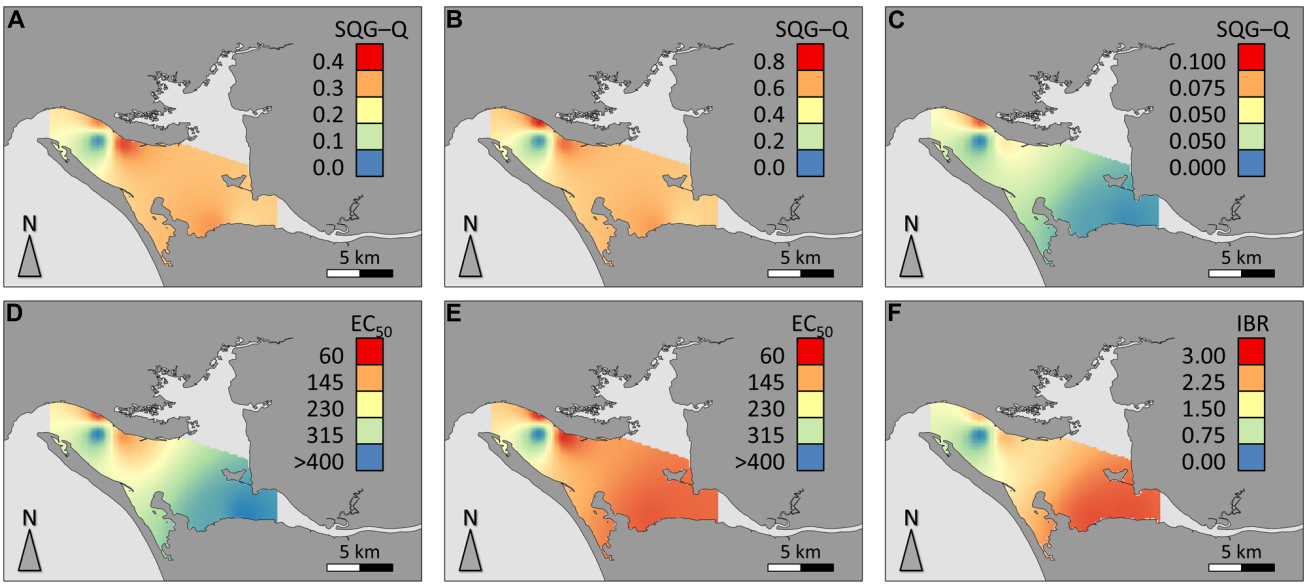

FIGURE 5 | Spatial distribution of data for the study area. (A) SQG-Q for total sediment contaminants (metals plus organic); **(B)** SQG-Q for sediment metals; **(C)** SQG-Q for organic sediment contaminants; **(D)** HepG2 EC_{50} for DNA strand breakage (crude extract exposure); **(E)** HepG2 EC_{50} for oxidative DNA strand breakage (crude extract exposure); **(F)** IBR for oxidative stress-related biomarkers, all species combined (clam, fish, and cuttlefish). SQG-Qs and IBR are dimensionless. EC_{50} estimates are expressed as mg SEQ/mL.

The current results are also accordant with those obtained by Kammann et al. (2004), who subjected a fish cell line (from *Cyprinus carpio*) to extracts (also transferred to DMSO) from marine sediments and observed that extracts obtained with more polar solvents were more genotoxic (as determined through the standard Comet assay) than those obtained with *n*-hexane. The same authors discussed that reduced metabolic activation could, at least in part, contribute to explain the results. As such, it is possible, though, that enhanced metabolic activation in HepG2 cells could have rendered more significant results for the tests with fractions 2 and 3 (prepared with more hydrophobic solvents) than actually measured (**Table 4**), even though these cells are generally acknowledged to retain the mechanisms involved in PAH bioactivation (with production of ROS as by-products) by CYP mixed-function oxidases (Knasmüller et al., 2004). However, inefficient extraction cannot be definitely excluded. The current results for fraction 1 (crude extract) are more indicative of metal-induced genotoxic effects (oxidative and non-oxidative), which is in good agreement with the results from the cluster analyses and the overall contamination pattern of sediments (**Figure 6**). It must also be noticed that cytotoxicity in HepG2 cells exposed to the different extracts was not clearly related to DNA damage, which is in accordance with other works dealing with *in vitro* exposures to whole marine sediment extracts (e.g., Yang et al., 2010). The results indicate that the complex mixture of toxicants within the tested sediments, specifically fractions 1 and 4, elicit differential genotoxic and cytotoxic effects. It must also be stressed that the cytotoxic effects of solvents may be disregarded since, in all cases, the solvents were evaporated and replaced with DMSO.

There are indications that the standard alkaline Comet assay may be less sensitive to detect PAH-induced DNA lesions when compared, for instance, to the determination of adduct formation, inclusively in HepG2 cells (Tarantini et al., 2009). This information may leads to the hypothesis that PAH-induced non-oxidative DNA damage might have been underestimated in HepG2 cells exposed to the crude extracts from sediments N1 and N2. Even so, the FPG-modified Comet assay has been found to greatly increase the assay's sensitivity when surveying environmental toxicants (Kienzler et al., 2012), which is accordant with the present findings (**Figure 3**; **Table 4**), particularly in HepG2 cells exposed to the crude and methanolic extracts. From the results, it may be inferred that sediment extract fractioning combined with the enzyme-modified Comet assay is a potentially valuable toxicity identification evaluation (TIE) strategy to monitor environmental genotoxicants, in the sense that by removing causative agents, cause–effect relationships may be sought through a break-down approach (see Chapman and Hollert, 2006). Nonetheless, this sort of methodology needs yet much research with respect to establishing causation, i.e., to determine toxicants and respective effects of exposure *in vitro* and *in vivo*.

Even though fish and mammalian cell lines have been found equally sensitive to test cytotoxic and genotoxic effects of environmental contaminants (Castaño and Gómez-Lechón, 2005), there are many differences between *in vitro* and *in vivo* bioassays that call for caution when direct comparisons are made, particularly if animals collected from the wild are being surveyed. Anti-oxidative stress responses in organisms are acknowledged to be complex and dependent of numerous factors, internal and external, of which toxicant concentrations in the environment account for just a few. Although the subject is not well understood in aquatic invertebrates, inhibition of anti-oxidant responses has been described in fish exposed to certain toxicants

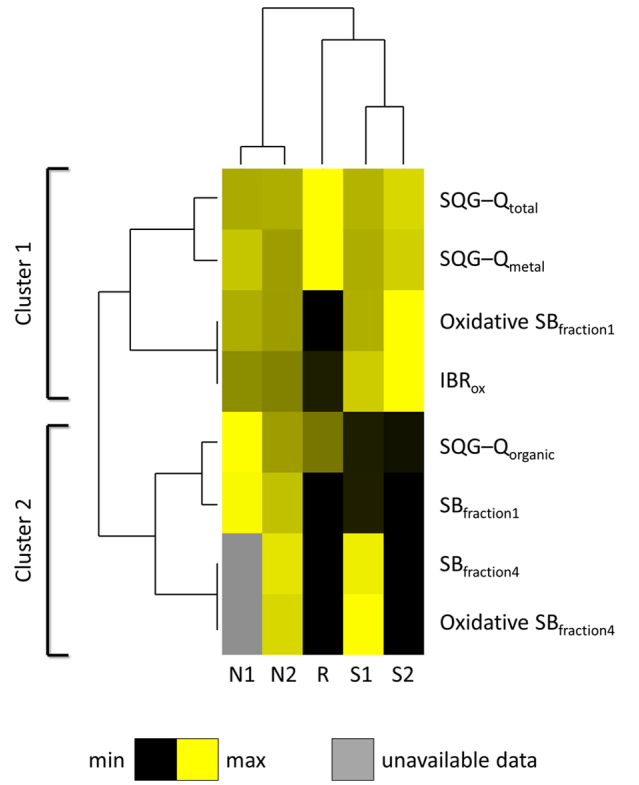

FIGURE 6 | Cluster analysis heatmap. Analysis combines sediment collection sites (N1, N2, S1, S2, and R) plus SQG-Q scores for sediment contaminants (total, metal, and organic pollutants) and biological responses: DNA strand breakage (SB), oxidative and non-oxidative, inputted as 1-EC_{50} relatively to the highest %DNA in tails from the study), for HepG2 cells exposed to fractions 1 (crude extract, DCM:methanol extraction) and 4 (methanol extraction only), plus IBR for oxidative stress biomarkers combining clam, fish, and cuttlefish (IBR_{ox}). Clustering between endpoints was achieved taking Spearman's rank-order correlation *R* as distance metric. Clustering between sites was obtained with Euclidean distances. Complete linkage as employed as amalgamation rule for the dendrograms.

(like metals) or their mixtures (e.g., Atli et al., 2006; Elia et al., 2007; Costa et al., 2010). This premise was also highlighted by Gonçalves et al. (2013), in face of elevated lipid peroxidation and higher level of histopathological alterations in the livers of sole collected from Sado 1. Moreover, previous studies have showed that sediments from this same area caused DNA strand breakage *in vivo* through a series of *in* and *ex situ* bioassays performed with *S. senegalensis*, which further supports the present findings (refer to Costa et al., 2008, 2011). It is also noteworthy that metals, the most representative contaminants in the estuary, may be indirectly genotoxic by impairing DNA repair and anti-oxidant enzymes (see Leonard et al., 2004), which likely affected HepG2 cells. Still, the integration of biomarker responses of the three species yielded differentiation between an impacted estuarine environment and the reference scenario, consistent with DNA damage measured through the Comet assay in HepG2 cells exposed to sediment extracts. Altogether, the present findings illustrate the purposefulness and adequacy of multiple lines-of-evidence in ERA, namely combining field sampling of multiple species, multiple biomarkers and *in vitro* assays to evaluate genotoxicity. As upheld by Chapman et al. (2013), the use of different lines-of-evidence, especially if appropriately incorporated into integrative weight-of-evidence assessments for management decision making, can reduce uncertainty and therefore assist determining causation.

CONCLUDING REMARKS

In the present work, an integrative assessment of genotoxic effects triggered by sediment-bound contaminants with oxidative stress biomarkers in three different species collected from an impacted estuary was conducted, consisting of an innovative combination of cell and whole-organism responses. The *in vitro* Comet assay (to determine oxidative or non-oxidative DNA damage) is an expanding tool in ERA, with the potential to become a LOE within its own right if proper validation through realistic case studies is achieved. Not dismissing the clear need to endeavor future research, the present work showed that the enzyme-modified Comet assay applied to HepG2 cells in a practical ERA context can yield results that are overall consistent and complementary with oxidative stress biomarkers analyzed in field-collected organisms. As such, the deployment of the *in vitro* Comet assay in human carcinoma cell lines and its combination with more traditional LOEs may meet its purpose even in scenarios where establishing cause–effect relationships is likely hampered by challenging circumstances such as the presence of complex mixtures of toxicants.

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The use of comet assay in plant toxicology: recent advances

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The systematic study of genotoxicity in plants induced by contaminants and other stress agents has been hindered to date by the lack of reliable and robust biomarkers. The comet assay is a versatile and sensitive method for the evaluation of DNA damages and DNA repair capacity at single-cell level. Due to its simplicity and sensitivity, and the small number of cells required to obtain robust results, the use of plant comet assay has drastically increased in the last decade. For years its use was restricted to a few model species, e.g., *Allium cepa, Nicotiana tabacum*, *Vicia faba, or Arabidopsis thaliana* but this number largely increased in the last years. Plant comet assay has been used to study the genotoxic impact of radiation, chemicals including pesticides, phytocompounds, heavy metals, nanoparticles or contaminated complex matrices. Here we will review the most recent data on the use of this technique as a standard approach for studying the genotoxic effects of different stress conditions on plants. Also, we will discuss the integration of information provided by the comet assay with other DNA-damage indicators, and with cellular responses including oxidative stress, cell division or cell death. Finally, we will focus on putative relations between transcripts related with DNA damage pathways, DNA replication and repair, oxidative stress and cell cycle progression that have been identified in plant cells with comet assays demonstrating DNA damage.

Keywords: plant comet assay, genotoxicity, metal, phytocompounds, radiation, pollutants, nanoparticles, DNA damages biomarkers

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Plant Comet Assay: General Considerations

The first reports on the use of comet assay in plants date from the 1990's (e.g., Cerda et al., 1993; Koppen and Verschaeve, 1996; Navarrete et al., 1997; Koppen and Angelis, 1998).

Despite similarities with other eukaryotic systems, namely animal models, the comet assay protocols for plants take into account relevant differences including the presence of a rigid cell wall in plant cells. The localized presences of characteristic meristematic regions (e.g., the concentration of highly dividing cells in the root apex) and the fact that root is usually the organ directly in contact with contaminated soil and water, have also influenced the establishment of plant comet assays in ecotoxicological approaches. Technical details concerning plant comet assays in different organs and species have been thoroughly reviewed by Gichner et al. (2009).

For almost a decade, the comet assay remained restricted to some toxicological studies and to a few model species including *Allium cepa, Nicotiana tabacum*, *Vicia faba,* and *Arabidopsis thaliana* (for review, Gichner et al., 2009; Ventura et al., 2013).

Plant comet assay has been applied to an increasing variety of adverse conditions. Some recent reviews on this subject (Gichner et al., 2009; Ventura et al., 2013) revised most relevant advances in plant comet assay up to 5 years ago. Since then an increasing interest for comet assay in plants was shown (136 articles published between 2010 and March 2015 vs. 89 between 1995 and 2009). Therefore, here we will mostly emphasize most relevant advances within the last 5 years, and highlight current applications of this technique in plant (eco) toxicological studies. We will also discuss advances on genetic studies involving DNA damage and repair.

Basic Principles and Methodologies

Comet assays traditionally use cell suspensions, which are embedded in agarose on a microscope slide, and exposed to lysis by exposure to detergent and high salt solutions (for review Collins et al., 2008; Azqueta et al., 2009). Lysis allows removing membranes and soluble cell components, leaving a supercoiled DNA nucleoid (Azqueta et al., 2011b). When submitted to electrophoretic conditions, DNA fragments will migrate toward the anode, forming a typical "comet tail." The amount of strand breaks is overall proportional to the amount of DNA in the tail respectively to the DNA remaining in the head (Hovhannisyan, 2010).

However, in plants, the presence of a cell wall causes technical issues for performing the comet assay on plant tissues. To overcome these problems, a simple and efficient mechanical extraction to isolate cell nuclei was developed by Cerda et al. (1993), and then improved by Koppen and Angelis (1998), Navarrete et al. (1997), and Gichner and Plewa (1998). Since then, most of the researchers used directly those protocols or derived versions, such as described in Gichner and Plewa (1998). Recently, Pourrut et al. (2015) identified the key steps of comet assay in plants and proposed an optimized protocol to increase its reliability and its throughput. In the case of plant chopping, particular attention has to be paid to the presence of chloroplasts as they are important sources of free radicals and oxidative damage. For example, the first article on plant comet assay testing chemicals used isolated nuclei of *Vicia faba* root cells (Koppen and Verschaeve, 1996). In cellular assays, plants exposed to suspected genotoxicants are processed for nuclei isolation and analysis, whereas in acellular assays, nuclei from non-stressed plants are isolated and then incubated with the genotoxicants, before comet assay analysis.

The use of protocol variants allows detecting a wide range of DNA damages (see for review Angelis et al., 1999; Collins et al., 2008). Briefly, an alkaline treatment (referred hereafter as A/A) and electrophoresis at pH 13 or higher allows the detection of most single and double DNA strand breaks (SSBs and DSBs) and also alkali-labile sites. When the unwinding and subsequent electrophoresis are performed using a buffer pH∼7–8, the comet assay is called "neutral" (N/N). A crucial difference is that at alkaline conditions, apurinic/apyrimidinic sites are more easily subjected to break (for details refer to Azqueta et al., 2011b). Other pH-variants (e.g., A/N) have meanwhile been introduced as alternative comet assays.

Moreover, the information provided by comets may also be increased by exposing the DNA to enzymes recognizing a specific lesion, e.g., formamidopyrimidine DNA glycosylase, Endonuclease III, thereby originating specific breaks. However, despite their strong interest and their early introduction in plant studies (Menke et al., 2000), these enzymes are still not much used in plants.

Comets may then be visualized by microscopy, by using a suitable DNA-binding dye, e.g., fluorescent dyes or silver staining. Data can be analyzed by visual scoring, ranging from 0 to 4 according to the damage class, or using computer-based image analysis (e.g., the software http://casplab.com/) that allows the quantification of several comet parameters, including the tail DNA %, tail length, tail extension moment or Olive tail movement (Azqueta et al., 2011b). Criteria for the best scoring approaches are however debatable (e.g., Azqueta et al., 2011a), but independently of the approach and scoring, it is consensual that this technique allows collecting data suitable for robust statistical analyses.

Radiation

Plants are prone to DNA damage upon exposure to radiation from natural or anthropogenic sources. For this reason, the analysis of DNA damage in irradiated plants is a topic of growing interest and sensitive methods for detection of DNA damage have been applied (**Table 1**).

The effects of light excess on plant DNA using comet assay were firstly investigated by Ojima et al. (2009) on *Raphanus sativus* protoplasts. These authors demonstrated that light excess causes DNA degradations mediated by oxidative stress. In 2010, Nishioka et al. confirmed the role of reactive oxidative species (ROS) in light excess-induced DNA damages in *Ipomoea aquatica* root protoplasts, and correlated DNA damages observed by comet assay with chlorophyll degradation. However, these two studies did not take into consideration the potential role of UV in light-induced DNA damages. In a study designed to investigate UV-A and UV-B effects, Jiang et al. (2007) performed comet to detect specific DNA lesions as well as pyrimidine dimers formation (using T4 endonuclease V) in irradiated *Spirodela polyrhiza* protoplasts. These results were confirmed later in *Arabidopsis thaliana* root tip cells (Jiang et al., 2009, 2011). Jiang et al. (2011) also demonstrated that UV-B-induced DNA damage results in the delay of G1-to-S transition of plant cell cycle. However, by using a neutral comet assay (N/N variant), Roy et al. (2011) showed that UV-B-induced lesions were reversible, particularly in *A. thaliana* wild-type (Col-0), compared to DNA polymerase λ UV-B sensitive mutants. UV-C was also shown to induce both SSBs and DSBs in *Arabidopsis plumbaginifolia* protoplasts (Abas et al., 2007). These authors also highlighted the usefulness of the comet assay as an analytical tool for the analysis of repair kinetics in protoplasts. These results were confirmed by Bilichak et al. (2014) on *A. thaliana* protoplasts.

Besides natural exposure to radiation, plants are also irradiated for industrial purposes. For example, gamma (γ)-rays are used to increase seed vigor and/or enhance plant tolerance to environmental stresses. Navarrete et al. (1997) pioneered the

TABLE 1 | Overview of comet assay studies in plant toxicology.

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
RADIATION								
Light	*A. thaliana*	Leaves	1300 μmol m^{-2} s^{-1}	Galbraith	A/A pH > 13	0.72 V/cm, 300 mA, 25 min, 4°C	(O)TM	Zeng et al., 2010
	I. aquatica	Roots	22 W m^{-2}	PBS	A/A pH > 13	25 V, 300 mA 10 min, 4°C	IN_D	Nishioka et al., 2010
	R. sativus	Cell suspension	430 W m^{-2}	PBS	A/A pH > 13	25 V, 300 mA 10 min, 4°C	IN_D	Ojima et al., 2009
UV	*A. thaliana*	Leaves	0.5 W m^{-2} UV-B, UV-A	Galbraith,T4endoV	A/A pH > 13	0.72 V/cm, 300 mA, 15 min, 4°C	(O)TM	Jiang et al., 2009
		Leaves	0.45 W m^{-2} UV-B, UV-A	Galbraith,T4endoV	A/A pH > 13	0.72 V/cm, 300 mA, 15 min, 4°C	(O)TM	Jiang et al., 2011
		Seedlings	3.5 kJm^{-2} UV-B	PBS-EDTA	N/N pH 8	1 V/cm, 12 mA, 5 min	%TD	Roy et al., 2011
	N. plumbaginifolia	Cell suspension	236 J mUV-C	PBS	A/A pH > 13	0.7 V/cm, 300 mA, 20 min	%TD	Abas et al., 2007
					N/N pH 8.4	2 V/cm, 10 mA, 2 min	%TD	Abas et al., 2007
	S. polyrhiza	Protoplasts	0.5 W m^{-2} UV-B, UV-A	Tris, T4 endoV	A/A pH > 13	0.72 V/cm, 300 mA, 15 min, 4°C	(O)TM	Jiang et al., 2007
γ-ray	(foodstuffs)	Various	5 kGy γ-ray	PBS Ca,Mg free	N/N pH 8.4	2 V/cm, 2 min	VS	Cerda et al., 1997
	(foodstuffs)	Various	10 kGy γ-ray	PBS-EDTA	N/N pH 8.4	0.66–0.83 V/cm, 300 mA, 5–40 min	%TD	Verbeek et al., 2008
	(foodstuffs)	Seeds	1 kGy γ-ray	PBS	N/N pH 8.4	2 V/cm, 2 min	TL	Koppen and Cerda, 1997
	A. cepa	Roots	4 Gy γ-ray	Sörensen(mod)	A/A pH > 13	0.65 V/cm, 230 mA, 20 min, 10°C	TL/HD	Navarrete et al., 1997
			50 Gy γ-ray	Tris	A/A pH > 13	0.65 V/cm, 230 mA, 20 min, 4°C	(O)TM	Saghirzadeh et al., 2008
	A. thaliana	Roots, leaves	3 Gy γ-ray	PBS	N/N pH 8.4	2 V/cm, 10 mA, 2 min, 4°C	VS	Vandenhove et al., 2010
		Seedlings	100 Gy γ-ray	PBS-EDTA	A/A pH 8.4	0.7 V/cm, 4°C	%TD	Moreno-Romero et al., 2012
	H. vulgare	Roots	110 Gy γ-ray	Sörensen(mod)	N/N pH 8	10 V/cm, 120 mA, 40 min, 4°C	%TD	Stoilov et al., 2013
					A/A pH 12.6	1 V/cm, 15 min, 4°C	%TD	Stoilov et al., 2013
	M. truncatula	Cell suspension	50 Gy γ-ray	Sörensen(mod)	N/N pH 8.4	1 V/cm, 8 min	VS	Donà et al., 2014
					A/A pH > 13	0.72 V/cm, 300 mA, 20 min, 4°C	VS	Donà et al., 2014
	N. tabacum	Roots, leaves	40 Gy γ-ray	Sörensen(mod)	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Gichner et al., 2000
	O. sativa	Seedlings	200 Gy γ-ray	PBS-EDTA	A/A pH > 13	0.72 V/cm, 20 min, 4°C	VS	Macovei and Tuteja, 2013
		Seeds	200 Gy γ-ray	PBS-EDTA	N/N pH 8.4	1 V/cm, 8 min	VS	Macovei et al., 2014
	P. x hybrida	Roots, leaves	100 Gy γ-ray	Sörensen(mod)	A/A pH > 13	0.72 V/cm, 300 mA, 20 min, 4°C	(O)TM	Donà et al., 2013
	P. nigra	Cell suspension	300 Gy γ-ray	Sörensen(mod)	A/A pH > 13	0.75 V/cm, 300 mA, 30 min, 4°C	%TD,LDR	Nishiguchi et al., 2012
	S. tuberosum	Roots, leaves	30 Gy γ-ray	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 15 min, 4°C	%TD	Gichner et al., 2008a
X-ray	*A. thaliana*	Leaves	15 Gy X-ray	PBS-EDTA	A/A pH > 13	300 mA, 15 min, 4°C	%TD	Enseit and Collins, 2015
	N. tabacum	Leaves, apex, Cotyledons	50 Gy X-ray	MBS-EDTA	A/N pH > 13/=8	1 V/cm, 10 mA, 5 min	%TD	Koppen et al., 1999
					N/N pH 8	2 V/cm, 10 mA, 2 min	%TD	Koppen et al., 1999
					A/A pH > 13	1 V/cm, 300 mA, 5 min, 4°C	%TD	Koppen et al., 1999
	O. sativa	Calli	100 Gy X-ray	Sörensen(mod)	N/N pH 8.4	1 V/cm, 8 min	(O)TM	Endo et al., 2012
	V. faba	Roots	50 Gy X-ray	MES saline	A/N pH > 13/=8	1 V/cm, 10 mA, 10 min	%TD	Koppen and Angelis, 1998
					A/N pH 12.5/=8	1 V/cm, 10 mA, 10 min	%TD	Koppen and Angelis, 1998

(Continued)

TABLE 1 | Continued

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
					A/A pH > 13	1 V/cm, 10 mA, 10 min	%TD	Koppen and Angelis, 1998
		Leaves,apex,	50 Gy X-ray	MBS-EDTA	A/N pH > 13/=8	1 V/cm, 10 mA, 10 min	%TD	Koppen et al., 1999
		Cotyledons			N/N pH 8	2 V/cm, 10 mA, 2 min	%TD	Koppen et al., 1999
					A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD	Koppen et al., 1999
METALS								
Monovalent	*V. faba*	Roots, leaves	50 mg/L $Tl(CH_3COO)$	Tris, C/A	A/A pH > 13	1 V/cm, 300 mA, 15 min, 4°C	(O)TM	Radić et al., 2009
Divalent	*A. cepa*	Roots	40 μM $CdCl_2$	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 25 min, 4°C	TL,(O)TM	Seth et al., 2008
			200 μM $CdCl_2$	Tris	A/A pH > 13	300 mA, 20 min	%TD	Arya and Mukherjee, 2014
	B. monnieri	Roots, leaves	500 μM $CdCl_2$	Tris, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Vajpayee et al., 2006
	L. sativa	Roots, leaves	50 μM $Cd(NO_3)_2$	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 30 min, 4°C	%TD,TL,(O)TM	Monteiro et al., 2012
	L. luteus	Roots	223 μM $CdCl_2$	Tris-$MgCl_2$	A/A pH 12.3	1 V/cm, 300 mA, 20 min, 8°C	TL	Arasimowicz-Jelonek et al., 2012
	N. tabacum	Roots, leaves	1.6 mM $CdCl_2$	Tris, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	%TD,(O)TM	Gichner et al., 2004
			15 μM $CdCl_2$	Tris	A/A pH > 13	0.8 V/cm, 300 mA, 20 min, 4°C	%TD	Tkalec et al., 2014
	P. sativum	Roots, leaves	7 (mg/kg) $CdCl_2$	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Hattab et al., 2010
	S. tuberosum	Roots, leaves	50 μM $CdCl_2$	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 15 min, 4°C	%TD	Gichner et al., 2008a
	V. faba	Roots	1 mM $CdCl_2$	Honda	A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD,TL,(O)TM	Koppen and Verschaeve, 1996
			200 μM $CdCl_2$	Tris	A/A pH > 13	300 mA, 15 min	%TD	Arya and Mukherjee, 2014
		Leaves	10 mg/L $CdCl_2 \cdot 2.5\ H_2O$	PBS-EDTA	A/A pH > 13	300 mA, 15 min, 4°C	VS	Lin et al., 2007
					A/N pH > 13/=8.4	4 min	VS	Lin et al., 2007
					N/N pH 8.4	15–17 mA, 6 min	VS	Lin et al., 2007
	V. unguiculata	Roots	10 mM $CdCl_2$	Tris-$MgCl_2$	A/A pH 12.3	1 V/cm, 300 mA, 20 min, 8°C	%TD,TL,(O)TM	Amirthalingam et al., 2013
	N. tabacum	Roots, leaves	50 μM $ZnCl_2$,15 μM $CdCl_2$	Tris	A/A pH > 13	0.8 V/cm, 300 mA, 20 min, 4°C	%TD	Tkalec et al., 2014
			80 mM $Zn(CH_3COO)_2$	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 25 min, 4°C	(O)TM	Procházková et al., 2013
	A. cepa	Roots	3 ppm $CuSO_4$, 11 ppm $CoCl_2$	Tris-$MgCl_2$	A/A pH 12.3	1 V/cm, 25 V, 20 min, 4°C	VS	Yıldız et al., 2009
			8 μM $CuSO_4$	Galbraith	A/A pH > 13	0.72 V/cm, 300 mA, 15 min, 4°C	(O)TM	Qin et al., 2015
	C. sativus	Roots	11 ppm $CuSO_4$	Tris-$MgCl_2$	A/A pH > 13	24 V, 300 mA, 30 min, 4°C	(O)TM	İşeri et al., 2011
	L. esculentum	Roots	60 ppm $CuSO_4$	Tris-$MgCl_2$	A/A pH > 13	24 V, 300 mA, 30 min, 4°C	(O)TM	İşeri et al., 2011
	M. truncatula	Leaflets	0.2 mM CuCl2	Sörensen(mod)	N/N pH 8.4	1 V/cm, 8 min	VS	Faè et al., 2014
	A. cepa	Roots	100 μM $Pb(NO_3)_2$	Galbraith	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4 °C	(O)TM	Jiang et al., 2014
			1 mM $Pb(NO_3)_2$	PBS	A/A pH > 13	25 V, 300 mA, 25 min	%HDNA, %TD, (O)TM	Kaur et al., 2014
	N. tabacum	Roots, leaves	2.4 mM $Pb(NO_3)_2$	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 25 min, 4°C	(O)TM	Gichner et al., 2008c
	T. triangulare	Roots	1.25 mM $Pb(NO_3)_2$	Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Kumar et al., 2013
	V. faba	Roots	20 μM $Pb(NO_3)_2$	PBS-EDTA, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	%TD	Pourrut et al., 2011b
Trivalent	*A. thaliana*	Roots	3 mM $B(OH)_3$	PBS-EDTA	N/N pH 8.4	1 V/cm, 15–17 mA, 6 min	%TD	Sakamoto et al., 2011
		Root	100 μM $AlCl_3$	PBS-EDTA	A/A	0.6 V/cm, 250 mA, 25 min	(O)TM	Nezames et al., 2012

(Continued)

TABLE 1 | Continued

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
	A. cepa	Roots	200 μM $AlCl_3$	PBS	A/A pH > 13	0.75 V/cm, 300 mA, 25 min, 4°C	TL	Achary et al., 2008
			800 μM $AlCl_3$	Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Achary and Panda, 2010
				Tris, C/A	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Achary et al., 2012a
				Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Achary et al., 2013
								Panda and Achary, 2014
	H. vulgare	Leaves	10 mM $AlCl_3$	Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM;VS	Achary et al., 2012b
	V. faba	Roots	1 mM $CrCl_3$	Honda	A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD, TL, (O)TM	Koppen and Verschaeve, 1996
Oxoanions	*A. cepa*	Roots	200 μM CrO_3	Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Patnaik et al., 2013
	P. sativum	Roots, leaves	2 g/L $K_2Cr_2O_7$	Tris	A/A pH > 13	0.74 V/cm, 15 min	%TD, (O)TM	Rodriguez et al., 2011
	V. faba	Roots, leaves	10 μM Na_2HAsO_4	PBS-EDTA	A/A pH > 13	300 mA, 15 min, 4°C	%TD, TL, (O)TM	Lin et al., 2008
		Roots	30 mg/L Na_2HAsO_4	Tris-NaCl	A/A pH > 13	25 V, 300 mA, 45 min	(O)TM	Boccia et al., 2013
			1 mM $K_2Cr_2O_7$	Honda	A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD, TL, (O)TM	Koppen and Verschaeve, 1996
NANOCOMPOUNDS								
MWCNT	*A. cepa*	Roots	50 mg/L MWCNT	Tris	A/A pH > 13	26 V, 300 mA, 20 min, 4°C	%TD,VS	Ghosh et al., 2011
			10 μg/L MWCNT	Tris	A/A pH > 13	26 V, 300 mA, 20 min, 4°C	%TD	Ghosh et al., 2015a
Metal NPs	*A. cepa*	Roots, leaves	80 mg/L Ag NPs	PBS	A/A pH > 13	25 V, 30 min, 4°C	%TD	Ghosh et al., 2012a
	B. rapa	Roots, leaves	10 mg/L Ag NPs	PBS-EDTA	A/A pH > 13	35 V, 300 mA, 25 min	%TD	Thiruvengadam et al., 2014
	N. tabacum	Roots, leaves	80 mg/L Ag NPs	PBS	A/A pH > 13	25 V, 30 min, 4°C	%TD	Ghosh et al., 2012a
Metal oxide NPs	*A. cepa*	Roots	100 ppm In_2O_3:SnO_2 NPs	Tris-$MgCl_2$	A/A pH > 12.3	1 V/cm, 25 V, 20 min, 4°C	VS	Ciğerci et al., 2015
			100 ppm Bi_2O_3 NPs	Tris-$MgCl_2$	A/A pH 12.6	1 V/cm, 25 V, 20 min, 4°C	VS	Liman, 2013
			100 mg/L TiO_2 NPs	Tris	A/A pH > 13	0.75 V/cm, 300 mA, 15 min, 4°C	(O)TM	Pakrashi et al., 2014
	L. esculentum	Roots	2 mg/ml NiO NPs	Galbraith, C/A	A/A pH > 13	0.7 V/cm, 300 mA, 30 min, 4°C	VS	Faisal et al., 2013
	N. tabacum	Roots, leaves	10 mM TiO_2 NPs	Tris	A/A pH > 13	26 V, 300 mA, 20 min, 4°C	%TD,VS	Ghosh et al., 2010
Quantum dots	*M. sativa*	Cell suspension	100 nM CdSe/ZnS QDs	MES $CaCl_2$/	A/N pH > 13/=8.4	25 V, 10 mA, 10 min, 4°C	VS	Santos et al., 2013
				FPG, EndoIII	N/N pH 8.4	25 V, 10 mA, 10 min	VS	Santos et al., 2013
ORGANIC POLLUTANTS								
Dyes	*A. cepa*	Roots	dyes of Petunia and	PBS, Tris	A/A pH > 13	0.7–0.75 V/cm, 300 mA, 20–25 min, 4°C	%TD,TL, %HDNA,(O)TM	Watharkar and Jadhav, 2014
			Gailardia	PBS, Tris	A/A pH > 13	0.7–0.75 V/cm, 300 mA, 20–25 min, 4°C	%TD,TL, %HDNA,(O)TM	Watharkar and Jadhav, 2014
Pesticides	*A. cepa*	Roots	100 ppm chlorfenvinphos 100 ppm fenbuconazole	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 25 min, 4°C	VS	Türkoğlu, 2012
			100 ppm fenaminosulf	Tris-MgCl2	A/A pH > 13	1 V/cm, 20 min, 4°C	VS	Liman et al., 2011

(Continued)

TABLE 1 | Continued

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
			80 ppm imazethapyr	Tris-MgCl2	A/A pH > 13	1 V/cm, 20 min, 4°C	VS	Liman et al., 2015
	I. balsamina	Leaves	145 nM feranimol	Sörensen(mod)	A/A pH > 13	0.66 V/cm, 230 mA, 10 min, 4°C	LDR,VS	Poli et al., 2003
	O. sativa	Calli	5 mg/L aphidicolin	PBS-EDTA	N/N pH 8.4	6 min	(O)TM,VS	Kwon et al., 2013
	P. vulgaris	Roots	0.3 ppm 2,4-D, 0.3 ppm Dicamba	Tris-$MgCl_2$	A/A pH 12.3	1 V/cm, 25 V, 20 min, 4°C	VS	Cenkci et al., 2010
Polyhalogenated	*A. cepa*	Roots	100 mg/L bromoform, 200 mg/L chloroform	Tris-$MgCl_2$	A/A pH 12.3	1 V/cm, 25 V, 20 min	VS	Khallef et al., 2013
	N. tabacum	Roots, leaves	4.8 mM CBA, 1 mM DCBA, 0.48 mM TCBA	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 25 min, 4°C	(O)TM	Gichner et al., 2008b
CONTAMINATED MATRICES								
Fly ash	*A. cepa*	Roots	fly ash mixtures (100%)	Tris	A/A pH > 13	0.7 V/cm, 300 mA, 20 min, 4°C	%TD,TL,(O)TM	Chakraborty et al., 2009
							%TD	Chakraborty and Mukherjee, 2011
			soil containing fly ash	Tris	A/A pH > 13	0.7 V/cm, 300 mA, 20 min, 4°C	%TD,(O)TM	Ghosh et al., 2012b
	C. occidentalis	Leaflets	soil containing fly ash	Sörensen(mod)	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	TL,VS	Love et al., 2009
	V. zizanioides	Roots	fly ash mixtures (100%)	Tris	A/A pH > 13	0.7 V/cm, 300 mA, 20 min, 4°C	%TD	Chakraborty and Mukherjee, 2011
Effluents	*A. cepa*	Roots	100% acid mine drainage	PBS-EDTA	A/A pH > 13	25 V, 300 mA, 20 min, 4°C	VS	Defaveri et al., 2009
								Netto et al., 2013
	L. minor	Plant	effluent waters	Tris	A/A pH > 13	1 V/cm, 300 mA, 20 min, 4°C	(O)TM	Radić et al., 2010
			fertilizer polluted water	Tris	A/A pH > 13	1 V/cm, 300 mA, 20 min, 4°C	%TD,(O)TM	Radić et al., 2013
Leachates	*A. cepa*	Roots	100% landfill leachate	Tris	A/A pH > 13	1 V/cm, 300 mA, 20 min	TL,(O)TM	Garaj-Vrhovac et al., 2013
	E. fetida	Shoots	100% landfill leachate	Tris	A/A pH > 13	25 V, 30 mA, 5 min, 4°C	(O)TM	Manier et al., 2012
	T. repens	Shoots	100% landfill leachate	Tris	A/A pH > 13	25 V, 30 mA, 5 min, 4°C	(O)TM	Manier et al., 2012
Metals	*N. tabacum*	Leaves	metal-polluted soil	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 25 min, 4°C	(O)TM	Gichner et al., 2006
	S. tuberosum	Leaves	metal-polluted soil	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 15 min, 4°C	(O)TM	Gichner et al., 2006
	T. repens	Leaves	metal-polluted soil	PBS	A/A pH > 13	300 mA, 15 min, 4°C	%TD, TL, (O)TM, VS	Bhat et al., 2011
Chemicals	*N. tabacum*	Leaves	PCB-polluted soil	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Gichner et al., 2007
Radiation	*A. cepa*	Roots	19000 Bcq/kg ^{226}Ra soil	Tris	A/A pH > 13	0.65 V/cm, 230 mA, 20 min, 4°C	(O)TM	Saghirzadeh et al., 2008
Gases	*P. tremuloides*	Leaves	1.5 × O_3, 200 ppm above normal CO2	PBS-EDTA	A/A pH > 13	1 V/cm, 300 mA, 30 min, 4°C	%TD	Tai et al., 2010
PHYTOCOMPOUNDS								
	A. cepa	Roots	100 mg/l *T. turcica* extract	Tris-$MgCl_2$	A/A pH > 13	1 V/cm, 20 min, 4°C	VS	Ciğerci et al., 2014
	L. sativa	Roots	200 μM epinodosin	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 25 min, 4°C	%TD,TL,(O)TM	Ding et al., 2010a

(Continued)

TABLE 1 | Continued

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
			200 μM rabdosin B	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 25 min, 4°C	%TD,(O)TM	Ding et al., 2010b
			5 μM narciclasine	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 25 min, 4°C	%TD,(O)TM	Hu et al., 2014
	P. alba	Roots	100 nM saponins	PBS	A/A pH > 13	0.72 V/cm, 20 min, 4°C	VS	Paparella et al., 2015
					N/N	1 V/cm, 8 min, 4°C	VS	Paparella et al., 2015
	R. sativus	Radicles	100% extract J. regia	Tris	A/A pH > 13	25 V, 300 mA, 20 min, 4°C	%TD,TL,(O)TM	Petriccione and Ciniglia, 2012
OTHERS								
Osmostressors	*A. thaliana*	Seedlings	200 mM NaCl	PBS-EDTA	N/N pH 8	1 V/cm, 12 mA, 5 min	%TD	Roy et al., 2013
	M. truncatula	Roots	50 g/L PEG 6000	Sörensen(mod)	N/N pH 8.4	1 V/cm, 8 min	VS	Confalonieri et al., 2014
	O. sativa	Seedlings	100 mM NaCl	PBS-EDTA	A/A pH > 13	0.72 V/cm, 20 min, 4°C	VS	Macovei and Tuteja, 2013
CONTROL MUTAGENS								
	A. cepa	Roots, leaves	8 mM EMS	Tris, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 20 min, 4°C	%TD	Bandyopadhyay and Mukherjee, 2011
	A. thaliana	Seedlings	50 mg/L BLM	PBS-EDTA	N/N pH 10	1 V/cm, 12 mA, 5 min	%TD	Böhmdorfer et al., 2011
			50 mg/L BLM	PBS-EDTA	N/N pH 8	1 V/cm, 12 mA, 5 min	%TD	Kozak et al., 2009
			8 mM MH	PBS-EDTA	A/A pH > 13	0.7 V/cm, 300 mA, 10 min, 4°C	%TD	Menke et al., 2001
			1 mg/L BLM, 5 mM MNU, 5 mM MMS, 8 mM MH, 0.5 mM MMC	PBS-EDTA	A/N pH > 13/=8.4	1 V/cm, 15–17 mA, 4 min	%TD	Menke et al., 2001
			1 mg/L BLM, 5 mM MNU, 5 mM MMS	PBS-EDTA	N/N pH 8.4	1 V/cm, 15–17 mA, 6 min	%TD	Menke et al., 2001
			2 μg/L BLM	PBS-EDTA	N/N pH 8.4	2 V/cm, 11 mA, 6 min	%TD	Wang et al., 2014
			2 μg/L BLM, 2 mM MMS	PBS-EDTA	A/N pH>13/=8.4	1 V/cm, 15–17 mA, 4 min	%TD	Waterworth et al., 2009
			2 μg/L BLM, 2 mM MMS	PBS-EDTA	N/N pH 8.4	0.6 V/cm,(20 V), 7 mA, 25 min	%TD	Waterworth et al., 2009
			50 μM BLM	PBS-EDTA	N/N pH 8.4	1 V/cm, room temperature	%TD	Moreno-Romero et al., 2012
	B. monnieri	Roots, leaves	100 μM MMS, 5 mM EMS	Tris, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Vajpayee et al., 2006
	C. capillaris	Leaves	2 mM MH	Tris	A/A pH > 13	15 V/cm, 340 mA, 15 min, 4°C	TL,(O)TM,VS	Kwasniewska et al., 2012
	H. vulgare	Roots	200 mg/L BLM	Sörensen(mod)	N/N pH 8	10 V/cm, 120 mA, 40 min, 4°C	%TD,VS	Georgieva and Stoilov, 2008
			100 mg/L BLM	Sörensen(mod)	A/A pH 12.6	1 V/cm, 15 min, 4°C	%TD,VS	Georgieva and Stoilov, 2008
	L. perenne	Leaves	60 mM EMS	Tris-EDTA	A/A pH > 13	0.72 V/cm, 300 mA, 5 min, 4°C	%TD	Pourrut et al., 2015
	M. giganteus	Leaves	60 mM EMS	Tris-EDTA	A/A pH > 13	0.72 V/cm, 300 mA, 5 min, 4°C	%TD	Pourrut et al., 2015
	N. tabacum	Roots, leaves	8 mM EMS	Tris, C/A	A/A pH > 13	0.72 V/cm, 300 mA, 20 min, 4°C	%TD	Bandyopadhyay and Mukherjee, 2011
		Roots	10 mM EMS, 20 μM H_2O_2	Tris, C/A	A/A pH>13	0.72 V/cm, 300 mA, 30 min, 4°C	(O)TM	Gichner, 2003b
		Leaves	4 mM EMS, 0.4 mM ENU, 0.5 mM MH	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 30 min, 4°C	%TD,(O)TM	Gichner, 2003a
			4 mM MH, 1 mM MNU	Tris	A/A pH > 13	16 V, 300 mA, 30 min, 4°C	%TD,(O)TM,VS	Juchimiuk et al., 2006
	P. x hybrida	Roots, leaves	3 mM EMS, 0.4 mM ENU	Sörensen(mod)	A/A pH > 13	0.72 V/cm, 300 mA, 20 min, 4°C	(O)TM	Donà et al., 2013
	P. patens	Protonema	50 mg/L BLM	PBS-EDTA	A/N pH > 13/=8.4	1 V/cm, 12 mA, 3 min	%TD	Holá et al., 2013
			50 mg/L BLM	PBS-EDTA	N/N pH 8.4	1 V/cm, 12 mA, 3 min	%TD	Holá et al., 2013

(Continued)

TABLE 1 | Continued

Stress	Species	Tissue	Maximum dose	Nuclei	Comet type	Electrophoresis	Analysis	References
			50 mg/L BLM	PBS-EDTA	N/N pH 8.4	1 V/cm, 12 mA, 5 min	%TD	Kamisugi et al., 2012
	S. tuberosum	Roots, leaves	8 mM EMS	Tris	A/A pH > 13	0.74 V/cm, 300 mA, 15 min, 4°C	%TD	Gichner et al., 2008a
	T. repens	Leaves	60 mM EMS	Tris-EDTA	A/A pH > 13	0.72 V/cm, 300 mA, 5 min, 4°C	%TD	Pourrut et al., 2015
	V. faba	Roots	1 mM MMS, 1 mM EMS, 0.1 μM MMC, 1 mM CH	Honda	A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD,TL,(O)TM	Koppen and Verschaeve, 1996
			50 M BrdU, 1 M FdU	MBS-EDTA	A/A pH > 13	1 V/cm, 300 mA, 10 min, 4°C	%TD	Koppen and Verschaeve, 2001
		Roots, leaves	5 mM EMS	Tris-EDTA	A/A pH > 13	0.72 V/cm, 300 mA, 5 min, 4°C	%TD	Pourrut et al., 2015
	Various species	Leaves	10 mM EMS	Tris	A/A pH > 13	0.72 V/cm, 300 mA, 15–30 min, 4°C	(O)TM	Gichner et al., 2003

C/A, cellular and acellular comets performed; A/A, alkaline variant; A/N, alkaline unwinding/neutral electrophoresis variant; N/N, neutral variant; IN_D; DNA intactness; %TD, % tail DNA; TL, tail length; (O)TM, (Olive) tail moment; VS, visual scoring; %HDNA, % head DNA; LDR, length of tail-to-DNA ratio; A. cepa, Allium cepa; A. thaliana, Arabidopsis thaliana; B. monnieri, Bacopa monnieri; C. occidentalis, Cassia occidentalis; C. capillaris, Crepis capillaris; C. sativus, Cucumis sativus; E. fetida, Eisenia fetida; H. vulgare, Hordeum vulgare; I. balsamina, Impatiens balsamina; I. Aquatica, Ipomoea aquatic; L. sativa, Lactuca sativa; L. minor, Lemna minor; L. perenne, Lolium perenne; L. luteus, Lupinus luteus; L. esculentum, Lycopersicum esculentum; M. sativa, Medicago sativa; M. truncatula, Medicago truncatula; M. giganteus, Miscanthus giganteus; N. plumbaginifolia, Nicotiana plumbaginifolia; N. tabacum, Nicotiana tabacum; O. sativa, Oryza sativa; P. x hybrida, Petunia x hybrid; P. vulgaris, Phaseolus vulgaris; P. patens, Physcomitrella patens; P. sativum, Pisum sativum; P. alba, Populus alba; P. nigra, Populus nigra; P. tremuloides, Populus tremuloides; R. sativus, Raphanus sativus; S. tuberosum, Solanum tuberosum; S. polyrhiza, Spirodela polyrhiza; T. triangulare, Talinum triangulare; T. repens, Trifolium repens; V. zizanioides, Vetiver zizanioides; V. faba, Vicia faba; V. unguiculata, Vigna unguiculata.

comet research in plants through the optimization of different steps in the comet assay applied to γ-irradiated *A. cepa* roots. Moreover, Cerda et al. (1997), Koppen and Cerda (1997) and Verbeek et al. (2008) optimized the comet assay to screen DNA damage in γ-irradiated seeds, dried fruits and spices. At the same period, Gichner et al. (2000, 2008a) used the A/A variant to study the effects of the γ-rays in irradiated tobacco and potato plants, respectively.

Later, Böhmdorfer et al. (2011) used this technique to study DSB formation in *Arabidopsis* homologous recombination deficient mutants subjected to γ-rays. On the other hand, Vandenhove et al. (2010) applied low γ-radiation dose rates for long periods to *Arabidopsis* plants. Despite the growth limitations and induction of oxidative stress response, the low applied radiation dose applied did not induce DNA damages measurable by the comet assay. Moreover, Macovei et al. (2014) demonstrated the occurrence of DSBs in rice (*Oryza sativa* L.) seedlings after exposure to γ-rays concomitant with a difference in expression profiles of three miRNAs, and an increase of reactive oxygen species (ROS) levels. Combining the use of the comet assay, and the expression of genes encoding DNA repair-related proteins, Nishiguchi et al. (2012) investigated the mechanisms of γ-radiation-induced DNA degradation and repair in Lombardy poplar (*Populus nigra* var. *italica*). Donà et al. (2014) studied further the mechanisms associated with plant sensitivity to γ-irradiation. By comparison of A/N and N/N variants of the comet assay in *Medicago truncatula*, these authors argued that active repair of DSBs occurred in treated cells. However, SSB repair did not occur and SSBs continued to accumulate as a consequence of increasing ROS levels. It is necessary to point that the distinction by comet assay of DSBs and SSBs is not trivial, since the neutral assay with prolonged protease digestion at high temperature will more likely only detect DSBs. The research team demonstrated in *Petunia x hybrida* treated with low and high-dose γ-irradiation that the level of DNA strand breaks was higher in the high-dose group. However, after 2 h the two groups showed identical amounts of strand breaks, suggesting a faster initial DNA repair in the high-dose group.

Alkaline and neutral DNA comet assays were also used to estimate both the levels of DNA damages and the repair potential in the barley lines T-1586 and D-2946 after exposure to γ-rays and Li ions (Stoilov et al., 2013). The authors found that the mutant line D-2946 was more sensitive to γ-radiation, supporting that susceptibility to this radiation is genotype dependent. Overall, these data support that the genotype, radiation dose and time of radiation exposure are crucial factors that determine the effects of radiation on DNA integrity.

In comparison to γ-rays, comet assay has been little used to evaluate DNA damages induced by X-rays. Using alkaline comet assay, Koppen and Angelis (1998) demonstrated that X-rays induce a linear increase of DNA content in the comet tail of irradiated *V. faba* plants. Endo et al. (2012) reported that X-ray exposure in *calli* of *Oryza sativa* resulted in a dose-dependent increase of DSBs, as shown by neutral comet assay. Recently, Enseit and Collins (2015) studied the effect of low dose radiations on DNA repair mechanisms using alkaline comet assay. They identified two phases of DNA repair after acute exposures of 5

and 15 Gy ("rapid" and "slow" phases). With lower exposures (2 Gy and lower), they also highlighted that "rapid" repair was so fast that it was difficult to detect.

Concerning radioactive contaminations, Saghirzadeh et al. (2008) successfully demonstrated that very high levels of natural radioactivity (e.g., by accumulation of ^{226}Ra) presented by soils were significantly genotoxic to *A. cepa* roots, with DNA damages measured by comet assay and compared to the effects of increasing γ-ray doses.

Metals

Most of the contaminated sites worldwide are contaminated with heavy metals. In Europe, heavy metals contaminated almost 50% of the investigated sites (Panagos et al., 2013). Exposure to metals may induce a variety of direct and indirect phytotoxic effects (e.g., Silva et al., 2010). In general metals induce more severe symptoms in roots than in leaves, since roots are in direct contact with the soil and generally with the toxic contaminant.

The first comet assays evaluating metal genotoxicity in plants were pioneered by Koppen and Verschaeve (1996) which studied chromium (Cr) and cadmium (Cd) genotoxicity in *V. faba*. These authors showed a dose-dependent increase in DNA damage. More recently, Cd-induced DNA degradations were also observed in *Trifolium repens* (Bhat et al., 2011), *Lactuca sativa* (Monteiro et al., 2012), *Lupinus luteus* (Arasimowicz-Jelonek et al., 2012), *Vigna unguiculata* (Amirthalingam et al., 2013), *N. tabacum* (Tkalec et al., 2014), *V. faba* and *A. cepa* (Arya and Mukherjee, 2014). However, dose-dependent responses were not clearly observed in these studies. This could be explained by the fact that these authors lead hydroponic studies and used very high and environmental-unrealistic concentrations of cadmium. Monteiro et al. (2012) suggested that these high concentrations could induce Cd-DNA adducts that lead to DNA-DNA/DNA-protein cross-links, and/or formation of longer DNA fragments, and/or impairment of DNA repair mechanisms, which could explain these results. Interestingly, the only study using soil spiked with environmental-realistic concentrations of cadmium (Hattab et al., 2010), demonstrated a dose-dependent increase in DNA damages in *P. sativum*. Tkalec et al. (2014) and Amirthalingam et al. (2013) also used the comet assay to understand Cd-induced genotoxicity mechanisms. They suggested the implication of oxidative stress while Arasimowicz-Jelonek et al. (2012) showed that scavenging the endogenous nitric oxide (NO) pool during Cd stress, despite reducing the programmed cell death, did not affect the degree of DNA damages evidenced by comet assay. Recently, comet assay was used to investigate the difference of sensitivity to Cd exposure of *A. cepa* and *V. faba* (Arya and Mukherjee, 2014). The results indicated that exposure to Cd induced slight dose-dependent increase in chromosomal aberrations, DNA fragmentation and micronucleus frequency in both *A. cepa* and *V. faba*. However, *V. faba* appeared more sensitive than *A. cepa* toward Cd-induced genotoxicity, which was correlated to the increased level of oxidative stress in root tissues.

Along with Cd, aluminum (Al) genotoxicity has been the most studied during the last years. Achary et al. (2008, 2012a) and Achary and Panda (2010) demonstrated dose-dependent DNA damage induced by Al exposure on *A. cepa* roots. These results were confirmed later on *Hordeum vulgare* (Achary et al., 2012b) and *Andropogon virginicus* (Ezaki et al., 2013). These studies also highlighted the implication of oxidative stress in Al genotoxicity. Comet assay was also used to investigate the mechanisms of Al genotoxicity, underscoring the role of cell wall-bound NADH-PX in the Al oxidative burst-mediated (Achary et al., 2012a), and the role of signal transduction mediated by Ca^{2+} (Achary et al., 2013) and MAP Kinases (Panda and Achary, 2014) in Al-induced cell death and DNA damage. Interestingly, these authors also described the occurrence of adaptation responses that involved oxidative stress, and that root cells conditioned with low doses of Al ($<10\,\mu M$ Al^{3+}) developed adaptive responses and protection mechanisms against genotoxic effects of the mutagenic agents methylmercuric chloride (MMCl) and ethyl methanesulfonate (EMS) (Achary et al., 2013). Moreover, the role of DNA damage in Al-dependent root growth inhibition was also investigated in *A. thaliana* mutants (Rounds and Larsen, 2008; Nezames et al., 2012).

The phytotoxicity of lead (Pb) including genotoxic aspects was reviewed by Pourrut et al. (2011a). Using comet assay, Gichner et al. (2008c) were the first to demonstrate dose-dependent Pb-induced DNA damage in *N. tabacum* in hydroponic and soil experiments. These results were confirmed on *Talinum triangulare* roots and correlated with Pb-induced oxidative stress (Kumar et al., 2013). However, both studies used very high and environmentally-unrealistic concentrations of Pb. More interestingly, dose-dependent Pb-induced DNA damage were also observed with lower and environmentally-realistic concentrations of Pb ($<20\,\mu M$ Pb) in *V. faba* plants (Pourrut et al., 2011b). Moreover, these authors also confirmed the role of oxidative stress in this damage process, since co-incubation with antioxidant vitamin E or the NADPH-oxidase inhibitor dephenylene iodonium inhibited DNA damage and micronuclei formation in exposed roots (Pourrut et al., 2011b). Recently, two studies performed on *A. cepa* confirmed the role of oxidative stress in lead-induced genotoxicity and that DNA damages are also tightly linked to the cell cycle (Jiang et al., 2014; Kaur et al., 2014).

Similarly, the micronutrient copper (Cu) was shown to induce significant DNA damages in *A. cepa* roots (Yıldız et al., 2009; Qin et al., 2015). Very high concentrations of copper chloride also increased DNA fragmentations in *P. sativum* roots but not in leaves (Hattab et al., 2010). Similarly to the above-cited metals, Cu-induced DNA damages were associated with cytotoxic damages involving oxidative stress in *Lycopersicon esculentum* and *Cucumis sativus* roots (İşeri et al., 2011) and other chromosome aberrations in *A. cepa* roots (Yıldız et al., 2009). Recently, Faè et al. (2014) used the neutral comet assay to demonstrate the overexpression efficiency of the DNA repair gene MtTdp2a for enhancing plant tolerance to Cu exposure in *Medicago truncatula* mutants.

By using the comet assay, Lin et al. (2008) proved that arsenate ($10\,\mu M$) induced DNA damages in *V. faba* leaves

and roots, in a dose-dependent manner and that these effects were associated with oxidative stress. Sturchio et al. (2011) confirmed As genotoxicity in *V. faba* roots grown on sandy and clay-loamy soil spiked with arsenate. In the same species, Boccia et al. (2013) combined the comet assay with infrared (FTIR), and near infrared (FTNIR) spectroscopy, to show that arsenate (20 and 30 mg/L) induced DNA damages which were associated with structural changes of different functional groups, suggesting the possible replacement of phosphate by arsenate in DNA.

The plant comet assay also contributed to clarify the effects of several other metals in plant DNA damages (**Table 1**). For example, Radić et al. (2009) demonstrated that the rare metal thallium (Tl), released to the environment as a by-product of Fe and Zn refining processes, induces DNA damages together with oxidative damages in *V. faba* seedlings. The comet assay was also helpful in demonstrating that boron (B) toxicity mechanism in plants involves DSBs and possibly replication blocks, with plant condensin II playing a critical role in DNA damages repair (Sakamoto et al., 2011). Rodriguez et al. (2011) and Rodriguez (2011) used a battery of genotoxic and cytotoxic biomarkers to assess Cr (VI) toxicity in pea, and were able to correlate Cr (VI)-induced DNA damages (demonstrated by comet assay) with cell cycle arrest at the G2/M checkpoint and with clastogenicity assessed by flow cytometry (Rodriguez, 2011, PhD thesis). Moreover, Patnaik et al. (2013) showed by alkaline comet assay that induction of DNA damage by Cr (VI) was dose-dependent in *A. cepa*. However, in plants exposed to 1-day treatment followed by 4-day recovery, no effects were found by comet assay. On the same plant species, cobalt (Co) was shown to induce significant DNA damages (Yıldız et al., 2009).

Besides some more established physiological analyses, the comet assay has also been conducted to determine the differential toxic effects affecting different plant organs. Procházková et al. (2013) showed that in *N. tabacum* zinc (Zn) induces higher DNA damages in roots compared to leaves. This differential effect was possibly attributable to the higher accumulation of Zn (II) in roots, compared to shoots. Tkalec et al. (2014) also observed these effects in *N. tabacum*. However, these authors also shown that, when Zn was added in the culture medium in combination with Cd, this metal conversely exhibited a protective effects against Cd-induced DNA damages.

It is worth noting that the interest of using the comet assay as a reliable biomarker on ecotoxicological assays is increasing, and Bandyopadhyay and Mukherjee (2011) applied both acellular and cellular comet tests to compare *A. cepa* and *N. tabacum* as toxicity models in rapid monitoring Cd-induced genotoxicity. Monteiro et al. (2012) used a battery of tests including the comet assay, to determine differences associated with organ dependence in Cd toxicity. The authors used *Lactuca sativa* and integrated cytostaticity/genotoxicity and oxidative stress data, where parameters measured by the comet assay (e.g., tail moment) were demonstrated to be relevant genotoxicity biomarkers. Despite still restricted to a few number, some studies have already used plant comet in field ecotoxicology assays of soils contaminated with metals (see Section "Contaminated Matrices" below).

Nanocompounds

Plant comet assays are also increasingly used to assess the phytotoxicity of small-scale materials (**Table 1**), e.g., nanomaterials and in particular nanoparticles (NPs). Nanomaterials possess unique properties suitable for a wide range of industrial applications. For this reason and due to their intense uses and subsequent release to the environment, they are currently classified as emerging contaminants. One example of emerging nanomaterials are carbon nanotubes, that depending on the physical properties can pose cytotoxicity to mammalian and plant cells (Ghosh et al., 2011). Ghosh et al. (2011, 2015a) demonstrated a correlation between DNA strand breaks and the concentration of multi-walled carbon nanotubes in *A. cepa*, supporting the genotoxic potential of this type of nanomaterials.

The increasing amount of NPs in groundwater and soil has raised environmental concerns regarding their putative toxicity and fate through food chains. A large group of NP contaminants include toxic or reactive metals NPs. One of the most relevant pioneer studies of NPs genotoxicity in plants was done with TiO_2 NPs in *A. cepa* (Ghosh et al., 2010). In this study the comet assay was used to assess DNA damages and this endpoint was combined with oxidative stress endpoints (e.g., malondialdehyde level). Moreover, in *A. cepa* roots, TiO_2 NPs induced DNA damages confirmed by comet assay and correlated with the occurrence of chromosomal aberrations (Pakrashi et al., 2014).

Silver nanoparticles (AgNPs) were shown to induce DNA damages in *A. cepa* and *N. tabacum* with more pronounced effects in roots than in shoots (Ghosh et al., 2012a).

Recently, using higher NPs concentrations, Thiruvengadam et al. (2014) also demonstrated a dose-dependent increase in DNA damages in *Brassica rapa* ssp. rapa, and this result was confirmed by DNA laddering and TUNEL assays.

Bismuth (III) oxide NPs increased the nuclear DNA damages in *A. cepa* plants. These data supported the concomitant observation of chromosomal aberrations and mitotic aberrations in the same tissues (Liman, 2013).

The alkaline comet assay showed an increase of DNA damages in tomato seedlings exposed to NiO-NPs up to 2 mg/ml (Faisal et al., 2013). In this study the authors also used the plant comet assay test to assess the percentage of necrotic and apoptotic cells, however, these conclusions must be regarded carefully as the validity of the comet assay in identifying apoptotic cells remains a matter of discussion (Collins et al., 2008).

Indium (III) oxide and tin (IV) oxide is a mixture widely used in industrial coating. A significant increase in DNA damages was recently observed of *A. cepa* root meristematic cells exposed to doses up to 100 ppm of indium tin oxide suspension (Ciğerci et al., 2015).

Besides metal oxide NPs, quantum dots form another type of nanomaterials increasingly prevalent in the environment. Quantum dots are nanomaterials used in electronics which possess semiconducting properties, composed for example of arsenic (As), selenium (Se) and tellurium (Te) in various proportions. Despite their increasing prevalence in the environment, the toxicity of quantum dots in plants is largely unknown. In a pioneer study, Santos et al. (2013)

used a battery of tests and gene expression related with DNA repair, and demonstrated that 10 nM 3-mercaptopropanoic coated-CdSe/ZnS quantum dots were cytotoxic and genotoxic to *Medicago sativa* cells. In this and other pioneer studies, the comet assay can play a pivotal role as a tool to assess environmental impacts of suspected emerging nanocontaminants.

Organic Pollutants

Several researchers have used the comet assay to monitor DNA damages induced in plants by numerous organic pollutants (**Table 1**). The most common organic chemical contaminants include reactive compounds, e.g., alkylating agents, azo dyes, cyclic aromatic hydrocarbons and chemicals incorporated in pesticides and herbicides.

The comet assay was recently used to better understand the role of homologous recombination and genome stability during DNA replication. Comet assay was used to study, in alfalfa, broad bean, lentil, miscanthus, onion, potato, tobacco, sugar beet and wheat, how different agents including ethyl methanesulfonate (EMS) and/or H_2O_2 induce DNA damages (Gichner et al., 2008a; Bandyopadhyay and Mukherjee, 2011; Pourrut et al., 2015). Due to their dose-dependent genotoxic effects, EMS and H_2O_2 became largely used as positive controls in plant comet assays, providing further robustness to the assay (Gichner et al., 2008a; Bandyopadhyay and Mukherjee, 2011; Pourrut et al., 2015). Similarly, the dose-dependent induction of DNA damages by compounds such as N-methyl-N-nitroso-urea (MNU), methyl methanesulfonate (MMS) and mitomycin C (MMC) (e.g., Menke et al., 2001; Juchimiuk et al., 2006) supported the wide use of these compounds as positive controls.

Azo dyes are important xenobiotic compounds, largely used in textile industry. Their putative genotoxicity was recently demonstrated in *Petunia grandiflora* and *Gaillardia grandiflora* by comet assay, in a pioneer study of plant–plant association for phytoremediation involving the treatment of textile dyes (Watharkar and Jadhav, 2014). Recently, it was demonstrated that bromoform (which may occur during disinfection processes of water) and chloroform (>25 μg/mL) increased chromosome aberrations and DNA damages, this last one assessed by comet assay in *A. cepa* roots (Khallef et al., 2013). Also chlorobenzoic acids (CBAs) may be found in soils contaminated with polychlorinated biphenyls (PCBs), and have mutagenic and carcinogenic effects in animals. Gichner et al. (2008b) demonstrated that the levels of CBAs inducing leaf withering or death also induced DNA migration in the comet assay.

In the last decade, several pesticides were demonstrated to induce DNA damages in plant cells (e.g., Poli et al., 2003). Endosulfan is an organochlorine pesticide widely used, and its genotoxicity was demonstrated in white clover (*Trifolium repens*) roots after exposure to doses up to 10 mg/L (Liu et al., 2009). The use of comet assay on *A. cepa* roots also demonstrated the genotoxic effects of the organophosphate insecticide/acaricide chlorfenvinphos and the triazole fungicide fenbuconazole (Türkoğlu, 2012). The experiment included tests/parameters such as the mitotic index, mitotic phase, chromosomal abnormalities, 2C DNA content (pg) and the plant comet assay on root meristem cells of *A. cepa*. Results indicated a robust negative correlation between both pesticides-induced DNA damage and 2C DNA amount. On the same plant model, Liman et al. (2011) studied the genotoxicity of the aromatic diazo fungicide and micro-biocide fenaminosulf. Comet assay clearly indicated a dose-dependent genotoxicity of Fenaminosulf in the root meristematic cells of *A. cepa*, which was confirmed by Mitotic index analysis. Herbicide genotoxicity was also evaluated by comet assay. Cenkci et al. (2010) demonstrated dose-dependent DNA-damages in common bean (*Phaseolus vulgaris*) roots used treated by two herbicides 2,4-D (2,4-dicholorophenoxyacetic acid) and Dicamba (3,6-dichloro-2-methoxybenzoic acid). These results were confirmed in the same study by RAPD analysis. Recently, Liman et al. (2015) also observed a dose-dependent DNA degradation induced by the imidazolinone herbicide Imazethapyr in *A. cepa* roots.

Antibiotics were also shown to induce DNA damages in plant cells. For example, the cytostatic effects of the antibiotic bleomycin (a DNA damaging glycopeptide) were demonstrated in plants, e.g., barley (Georgieva and Stoilov, 2008; Stoilov et al., 2013). Bleomycin also induced DNA oxidative damages and single and double strand breaks in the wild moss *Physcomitrella* lines and in the *lig4* mutant (Holá et al., 2013). Similarly, MMC induced a dose-dependent increase in DNA damages in *Arabidopsis* plants (Menke et al., 2001).

Contaminated Matrices

Despite the promising data concerning the robustness and suitability of the comet assay for screening metal-induced DNA damages in plant cells, its use to assess the genotoxicity of poly-contaminated matrices, including samples of contaminated soils, of leakages or fly ashes, remains scarce (**Table 1**). In a pioneer study, Gichner et al. (2006) used the alkaline comet assay to demonstrate DNA damages in both *N. tabacum* and *Solanum tuberosum* plants exposed to soils contaminated with a mixture of Cd, Cu, Pb, and Zn. Also, soil samples polluted with polychlorinated biphenyls were shown to induce DNA damages in tobacco plants (Gichner et al., 2007). These authors concluded that comet assays may be used for monitoring the DNA-damaging effects of environmental pollutants.

In a microcosm study, and using *T. repens* as plant model, Manier et al. (2012) found a dose-dependent increase in DNA damages in plants exposed to soil contaminated with landfill leachate. Garaj-Vrhovac et al. (2013) used the comet assay to validate two new methods of leachate treatment, which induced less DNA damages in *A. cepa* roots than the untreated landfill leachate. Comet assay was also used to evaluate the efficiency of new treatment technology to decrease acid mine drainage genotoxicity. Defaveri et al. (2009) and Netto et al. (2013) used *A. cepa* roots, and different biomarkers including DNA damages and other cytotoxic and physiological biomarkers, while Radić et al. (2010) used the aquatic species *Lemna minor*. In a previous study, these authors demonstrated in *Lemna minor* that the tail moment assessed by the plant comet assay and parameters related to oxidation were suitable as biomarkers for environmental monitoring of the toxicity of industrial effluents in Croatia (Radić

et al., 2010). Importantly, the same group (Radić et al., 2013) found comparable responses in fish and *Lemna minor* regarding DNA damage and oxidative stress, after exposure to polluted surface water contaminated by a fertilizer factory effluent rich in fluorides, metals, and polycyclic aromatic hydrocarbons. The authors highlighted that their results imply that conventional chemical analysis should be extended to genotoxicity/toxicity biological assays to better predict potential health hazard.

Fly ashes are generated during combustion, and include fine particles, with different sizes, rising to the atmosphere. Their complex constitution raised questions on their genotoxicity to animals and plants. Love et al. (2009) demonstrated, based upon comet assay results, that higher levels of DNA damages were found in leaves of *Cassia occidentalis* exposed to fly ash, compared to non-exposed controls. The authors suggested that these DNA damages might be associated with foliar concentrations of As and Ni absorbed from the fly ash. Ghosh et al. (2012b) studied the genotoxicity in *A. cepa* of soil samples contaminated with metal-rich fly ashes from a thermal power plant in India and concluded that the observed DNA damages could be correlated to the presence of toxic metals. Also, Chakraborty et al. (2009) studied the genotoxic effects of fly ash comparing the comet assay and the Allium test in this model species. The authors supported the combination of these two techniques in monitoring assays. The same group used the comet to validate the relevance of *Vetiveria zizanioides* as a good candidate for remediation of fly ash dumpsites (Chakraborty and Mukherjee, 2011). They demonstrated this plant could grow in the presence of fly ash without any genotoxic effects in comparison to A. cepa which exhibited a very high DNA degradation (>80%). Later, this research group used comet assay on *A. cepa* to monitor the remediation efficiency of *V. zizanioides* on fly ash amended soils (Ghosh et al., 2015b). They showed that this plant was able to strongly mitigate the genotoxic potential of these soils. These results were also confirmed by a reduction in micronuclei formation, binucleate cells and chromosomal aberrations.

The effects of air contaminants on plant DNA-damages have also been studied in the last years. For example, *Populus tremuloides* clones exposed to air enriched with O_3 alone, or $CO_2 + O_3$ showed increased DNA damages levels above background as measured by the comet assay, but these effects were genotype dependent (Tai et al., 2010).

Phytocompounds

A wide number of phytocompounds (including alkaloids, phenolic compounds, glycosides, flavonoids, anthocyanins, etc) may have cytotoxic and genotoxic effects or have protective roles against stressing conditions in a wide number of species, including humans. The way phytocompounds influence oxidative stress balances, and regulate programmed cell death pathways and cell cycle chekpoints, support their wide therapeutic use (e.g., Ascenso et al., 2013; Ferreira de Oliveira et al., 2014). Recently, the interest of using comet assay to monitor genotoxic effects of some phytocompounds on other plant species has emerged (**Table 1**). For example, Petriccione and Ciniglia (2012) demonstrated the occurrence of a dose-dependent accumulation of DNA damages in *Raphanus sativus* (radish) radicles treated with *Juglans regia* husk water extracts. It should be noted that the authors stressed the need of performing accurate and appropriate statistical evaluations of comet results, an emerging topic of discussion. Ciğerci et al. (2014) also used alkaline comet assay to demonstrate the genotoxicity of *Thermopsis turcica* extracts on *A. cepa* roots. They showed dose-dependent DNA damages which were confirmed by RAPD profile analysis.

The alkaloid narciclasine (extracted from *N. tazetta*) was recently shown to inhibit plant growth of *Oryza sativa*, *A. thaliana*, *Brassica rapa* or *Lactuca sativa* (Hu et al., 2014). The comet assay, complemented with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, showed a narciclasine dose-effect response in lettuce seedlings, and this triggered DNA damages may involve increased oxidative stress (Hu et al., 2014). Contrarily, anthocyanins protected DNA integrity (detected by comet assay) in *Arabidopsis* plants during prolonged exposure to high-light (1300 mmol/m^2/s) (Zeng et al., 2010).

Epinodosin, and rabdosin B, diterpenoids isolated from *Isodon japonica*, exhibited a biphasic dose-dependent effect on *Lactuca sativa* root growth. The inhibitory effects of both compounds found at higher doses was paralleled with an increase of DNA damages and an inhibition of root cell mitotic activity or retardation of the cell cycle, respectively (Ding et al., 2010a,b). Other terpenes (saponins) extracted from *Medicago sativa* were shown to induce SSBs and DSBs in *Populus alba* cell cultures (Paparella et al., 2015). Very interestingly, these authors demonstrated that for all 11 tested saponins, neutral comet assay resulted in similar DSBs patterns, indicating a general response to saponin-induced genotoxic stress, not related to the specific structure of these molecules. Differently, the evaluation of DNA damages performed with alkaline comet assay provided distinct profiles depending on the tested saponin.

Comet assay was also used to evaluate the effect of the phytohormone salicylic acid. Interestingly, Yan et al. (2013) demonstrated that salicylic acid can generate DNA damages in the absence of a genotoxic agent in *A. thaliana*, supporting that activation of DNA damage responses is an intrinsic component of the plant defense responses.

Comet Assay and Putative Genetic Associations

The comet assay has contributed to elucidate the DNA repair mechanisms involved in the response to external stress factors. A variety of methodologies can be used to investigate DNA repair mechanisms in plants (Azqueta et al., 2009), the most common being the study of plants exposed to DNA mutagens and comparison of plant strains deficient in specific DNA repair pathways. Ionizing radiation and a variety of genotoxins specifically induce DSBs and are frequently analyzed together with the action of radiomimetic compounds, such as bleomycin (e.g., Menke et al., 2001; Waterworth et al., 2009; Böhmdorfer et al., 2011; Wang et al., 2014), zeocin (Nishiguchi et al., 2012), or MMS (e.g., Menke et al., 2001; Vajpayee et al., 2006; Waterworth et al., 2009). Other mutagens frequently used to study DNA repair and strand breaks include agents that induce point mutations,

e.g., N-ethyl-N-nitrosourea (ENU), MNU, or EMS (e.g., Menke et al., 2001; Donà et al., 2013), and the DNA crosslinking agent MMC (e.g., Koppen and Verschaeve, 1996; Menke et al., 2001).

In the past, plant strains deficient in DNA repair pathways have been analyzed by comet assay for their DNA repair capability under specific genotoxic stress. The first observation of biphasic DSB repair in plants with extremly rapid first phase was by Kozak et al. (2009). This approach, led to the identification of *A. thaliana AtRad18* (*SMC6B*) and *AtRad21.1* (*SYN2*) as important effectors in early repair of DSBs, after treatment with bleomycin (Kozak et al., 2009). Also important, through the use of comet functional assays, Moreno-Romero et al. (2012) showed that *Arabidopsis* mutant plants quickly repaired the DNA damage produced by bleomycin and γ-rays, and that they showed preferential use of non-conservative mechanisms. Moreover, in *Arabidopsis* knock-down strains of DNA ligase I, Waterworth et al. (2009) found by neutral comet assay that the *LIG1* knock-down strains were less efficient in the repair of DSBs compared to wild-type, suggesting that the *AtLIG1* gene is involved also in DSB repair pathway.

Several transcripts related with DNA damage pathways, DNA replication, and repair, oxidative stress and cell cycle progression have been identified in plant cells associated with alterations in comet assay profiles. Some of the most relevant studies in wildtype plants are summarized in **Table 2**. For example, Endo et al. (2006) demonstrated that *Arabidopsis fas* mutants showed increased levels of DNA DSBs. The authors proposed that the induction of DNA DSBs and enhanced transcription of genes involved in Homologous Recombination (HR) might occur during S phase and stimulate HR in fas mutants. Also, levels of formed DSBs were compared in rice wild type plants vs. an aphidicolin-sensitive phenotype. Without aphidicolin treatment, both WT and *osrecql4-2* mutants produced very low levels of DSBs, but these increased in the mutants after treatment (Kwon et al., 2013).

Böhmdorfer et al. (2011) studied the involvement of γ-irradiation and MMC induced one protein (GMI1), a structural-maintenance-of-chromosomes-hinge domain-containing protein in mechanisms of somatic homologous recombination in *Arabidopsis* mutant lines. Comet assay demonstrated that the *gmi1* mutants had a reduced rate of DNA DSB repair during the early recovery phase after exposure to bleomycin. Also Yao et al. (2013) used the comet assay to show an increase of DNA damage levels in *Arabidopsis sdg2* mutants, containing a mutation at SET DOMAIN GROUP 2, necessary for global genome wide deposition of histone H3 lysine 4 trimethylation in chromatin. With these results, authors contributed to elucidate the regulation of SDG2-mediated H3K4me3 on chromatin structure and genome integrity in plants.

Sakamoto et al. (2011) studied *Arabidopsis* mutants (*heb1-1* and *heb2-1*) hypersensitive to excess of boron (B). Excess of B induced DNA damages and affected the expression of *HEB1* and *HEB2*, which encode respectively the CAP-G2 and CAP-H2 subunits of the condensin II protein complex, important in maintenance of chromosome structure. These results suggested that DSBs are a cause of B toxicity and that condensin II reduces the incidence of DSBs (Sakamoto et al., 2011).

Santos et al. (2013) demonstrated in *Medicago sativa* that exposure to increasing concentrations of MPA-CdSe/ZnS quantum dots, led to an increase of DNA damages, and up-regulated the transcription of the DNA repair enzymes formamidopyrimidine DNA glycosylase, tyrosyl-DNA phosphodiesterase I and DNA topoisomerase I.

Roy et al. (2011, 2013) reported that *Arabidopsis atpolλ* mutant lines exposed to UV-B radiation or to high salinity and MMC treatment s showed higher accumulation of DSBs than wild-type plants and a delayed repair of DSBs. This fact suggested the requirement of Pol λ in DSB repair in plants. Gamma irradiated *Populus nigra* suspension-cultured cells showed increased levels of DNA damage and increase of the transcripts *PnRAD51*, *PnLIG4*, *PnKU70*, *PnXRCC4*, and *PnPCNA* while *PnOGG1* mRNA was repressed (Nishiguchi et al., 2012). On the other hand, Donà et al. (2013) tested genotoxic effects of γ-irradiation and found significant fluctuations on the levels of DSB and different capacities of DNA repair, together with dose-rate-dependent changes in the expression of the genes *PhMT2* (encoding for a type 2 metallothionein) and *PhAPX* (encoding for a cytosolic isoform of ascorbate peroxidase).

Probing FISH techniques have been successfully applied to comet assay preparations to detect specific DNA lesions, nuclear organizer regions (NORs) and telomeric regions in *V. faba* (Menke et al., 2000) or 5S/25S rDNA in *Crepis capillaris* (Kwasniewska et al., 2012).

Salt, drought and osmotic stress are ever more emerging as abiotic defies intimately related with soil overuse and climate changes (e.g., Santos et al., 2002; Brito et al., 2003). Salt stress induction of DNA damages has been explored in e.g., *Arabidopsis* mutants by Roy et al. (2013) who supported the role of Polλ in DNA damages repair. Salt stress and/or radiation induction of DNA damages was studied in rice by Macovei and collaborators who also evaluated the expression of *OsXPB2*, *OsXPD*, *OsTFIIS*, and *OsTFIIS*-like genes (Macovei and Tuteja, 2013; Macovei et al., 2014). Recently, Balestrazzi et al. (2014) demonstrated in *Medicago truncatula* plants that a prolonged exposure to osmotic stress can cause unwanted DNA damages, while negatively affected the expression profiles of genes involved in DNA repair, namely *MtTdp1* (tyrosyl-DNA phosphodiesterase), *top1* (DNA topoisomerase I), *MtTFIIS* (transcription elongation factor II-S) and *MtTFIIS*-like. So, despite comet assay has not been consistently applied to these environmental stresses in plants, the available data of their interference with DNA integrity, opens a perspective of their use in the near future. Also, Confalonieri et al. (2014) demonstrated that in *Medicago truncatula* the MtTdp2α-gene overexpression prevented the accumulation of DSBs in absence or presence of osmotic stress, and that the *MtMRE11*, *MtRAD50* and *MtNBS1* genes that are involved in DSB sensing/repair, being up-regulated in the *MtTdp2α*-overexpressing plants grown under physiological conditions, were no further up-regulated under osmotic stress (Confalonieri et al., 2014).

Conclusions

In this review we have highlighted most relevant studies that used comet assay in plants to study the impact of stress conditions on

TABLE 2 | Genes differentially expressed in comet assay positive plants.

Gene	Gene function	Expr.	Stress	Species	References
BRCA1	HR—DSB repair, ATM pathway (DSB-inducible)	Up	γ-ray BLM boric acid	*A. thaliana* *A. thaliana* *A. thaliana*	Böhmdorfer et al., 2011 Wang et al., 2014 Sakamoto et al., 2011
CAP-G2 (HEB1)	Tolerance to DSB induction	Up	boric acid	*A. thaliana*	Sakamoto et al., 2011
CAP-H2 (HEB2)	Tolerance to DSB induction	Up	boric acid	*A. thaliana*	Sakamoto et al., 2011
FPG	BER; removal of oxidized purines	Up	CdSe/ZnS quantum dots	*M. sativa*	Santos et al., 2013
GMI1	HR—DSB repair, ATM pathway (DSB-inducible)	Up	γ-ray, BLM, MMC	*A. thaliana*	Böhmdorfer et al., 2011
GR1	HR—DSB repair, ATM pathway (DSB-inducible)	Up	BLM boric acid	*A. thaliana* *A. thaliana*	Wang et al., 2014 Sakamoto et al., 2011
KU70	NHEJ—DSB repair	Up	γ-ray, zeocin	*P. nigra*	Nishiguchi et al., 2012
KU80	NHEJ—DSB repair	Up	salt stress (NaCl)	*A. thaliana*	Roy et al., 2013
LIG4	NHEJ—DSB repair	Up	γ-ray, zeocin salt stress (NaCl)	*P. nigra* *A. thaliana*	Nishiguchi et al., 2012 Roy et al., 2013
OGG1	BER; removal of 7,8-dihydro-8-oxoguanine	Down	γ-ray	*P. nigra*	Nishiguchi et al., 2012
PARP1	DSB repair (ATM pathway); SSB repair (ATR pathway)	Up	boric acid	*A. thaliana*	Sakamoto et al., 2011
PCNA	DNA replication and repair	Up	γ-ray	*P. nigra*	Nishiguchi et al., 2012
Polλ	NHEJ; NER in response to UV; DNA replication	Up	UV-B salt stress (NaCl)	*A. thaliana* *A. thaliana*	Roy et al., 2011 Roy et al., 2013
RAD51	HR—DSB repair, ATM pathway (DSB-inducible)	Up	γ-ray, zeocin boric acid	*A. thaliana* *P. nigra* *A. thaliana*	Böhmdorfer et al., 2011 Nishiguchi et al., 2012 Sakamoto et al., 2011
RAD51A2	HR	Up	X-ray	*O. sativa L.*	Endo et al., 2012
TDP1β	Repair of topoisomerase I-mediated damages	Up	CdSe/ZnS quantum dots	*M. sativa*	Santos et al., 2013
TOP1β	Remove DNA supercoils: transcription, DNA replication, recombination	Up	CdSe/ZnS quantum dots	*M. sativa*	Santos et al., 2013
XRCC4	NHEJ—DSB repair	Up	γ-ray salt stress (NaCl)	*P. nigra* *A. thaliana*	Nishiguchi et al., 2012 Roy et al., 2013
APX	Detoxification of peroxide	Up	CdSe/ZnS quantum dots γ-ray	*M. sativa* *Petunia x hybrida*	Santos et al., 2013 Donà et al., 2013
SOD	Detoxification of superoxide	Up	CdSe/ZnS quantum dots	*M. sativa*	Santos et al., 2013
MT2	Metal binding, ROS radical neutralization	Up	γ-ray	*Petunia x hybrida*	Donà et al., 2013
CDKA1	Cell cycle regulation	Up	boric acid	*A. thaliana*	Sakamoto et al., 2011
CYCA2;1	Cell cycle progression	Up	boric acid	*A. thaliana*	Sakamoto et al., 2011

ATM, Ataxia telangiectasia mutated; ATR, ATM and Rad3 related; BER, base excision repair; HR, homologous recombination; NER, nucleotide excision repair; NHEJ, non-homologous end joining; DSB, double strand breaks; A. thaliana, Arabidopsis thaliana; M. sativa, Medicago sativa; O. sativa, Oryza sativa; P. nigra, Populus nigra.

plant DNA damages. This work was mostly focused on the most recent major advances in the last five, regarding conventional and emerging contaminants and complex matrices. The recent advances in the use of the plant comet assay to both a larger number of plant species, and a larger number of conditions, support the use of this technique as a robust and sensitive technique to assess DNA damages induced by stress conditions. Data also support that this simple and robust technique may be a powerful tool to complement conventional and -omics tools in situ environmental pollution monitoring. Moreover, new fields of research using plant comet assay are open, not only in environmental studies, but also in plant physiology, as this technique may help elucidating pathways involved in plant development, cell cycle/programmed cell death, or even plant disease resistance. Also, it remains an important field of research deciphering genetic mechanisms underlying processes related with DNA damage/repair, in which comet assay will have undoubtedly a crucial role.

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Comet assay to measure DNA repair: approach and applications

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Cellular repair enzymes remove virtually all DNA damage before it is fixed; repair therefore plays a crucial role in preventing cancer. Repair studied at the level of transcription correlates poorly with enzyme activity, and so assays of phenotype are needed. In a biochemical approach, substrate nucleoids containing specific DNA lesions are incubated with cell extract; repair enzymes in the extract induce breaks at damage sites; and the breaks are measured with the comet assay. The nature of the substrate lesions defines the repair pathway to be studied. This *in vitro* DNA repair assay has been modified for use in animal tissues, specifically to study the effects of aging and nutritional intervention on repair. Recently, the assay was applied to different strains of *Drosophila melanogaster* proficient and deficient in DNA repair. Most applications of the repair assay have been in human biomonitoring. Individual DNA repair activity may be a marker of cancer susceptibility; alternatively, high repair activity may result from induction of repair enzymes by exposure to DNA-damaging agents. Studies to date have examined effects of environment, nutrition, lifestyle, and occupation, in addition to clinical investigations.

Keywords: DNA repair, animal studies, human biomonitoring, occupational studies, clinical studies, base excision repair (BER), nucleotide excision repair (NER), comet assay

THE IMPORTANCE OF MEASURING DNA REPAIR

DNA is a molecule prone to damage from exogenous and endogenous sources with important consequences for mutagenic and carcinogenic processes. Cells possess repair systems that amend virtually all the damage before genome change can occur; repair mechanisms therefore play a crucial role in prevention of cancer. Different pathways, involving numerous groups of repair enzymes, deal with the various types of DNA damage (Friedberg et al., 2006, **Table 1**): insertion of one or a few bases followed by ligation deals with single-strand breaks (SSBs) in the sugar–phosphate backbone; homologous recombination and non-homologous end-joining deal with the more serious double-strand breaks (DSBs) in the sugar–phosphate backbone; base excision repair (BER) deals with small base alteration such as alkylation or oxidation; nucleotide excision repair (NER), the most complex repair pathway, deals with bulky adducts of different molecules covalently linked to bases, covalent bonds between adjacent bases in the same strand (intra-strand cross links), DNA–protein cross links, as well as covalent bonds across the double helix (inter-strand cross links); and finally mismatch repair deals with wrongly paired bases. All of these pathways are likely to be regulated in a different way. For instance, enzymes playing roles in BER are assumed to be constitutive since they deal with the oxidized bases produced as a result of the inevitable presence of reactive oxygen species (a by-product of respiration) while enzymes involved in NER are more likely to be inducible since they deal with lesions that are caused sporadically by exogenous agents (e.g., food mutagens, UV light).

DNA repair activity or potential is regarded as a valuable marker of susceptibility to mutation and cancer. Frequently, it is determined at the level of transcription by using DNA microarray techniques or by RT-PCR for selected genes involved in the different repair pathways. However, it is well known that the activity of an enzyme does not just depend on the rate of transcription and translation, and not even on the amount of protein present. Indeed, BER gene expression has been shown not to correlate with enzyme activity (Paz-Elizur et al., 2007), and so a phenotype assay seems to be more relevant. The comet assay has been widely used for measuring the repair activity of cells, and in the past decade also of tissues.

THE ALKALINE COMET ASSAY TO MEASURE DNA REPAIR

The alkaline comet assay, in its standard version, detects DNA strand breaks (SBs) and alkali-labile sites (ALS). This technique is based on the electrophoresis of single nucleoids (DNA attached to the nuclear matrix after cell lysis and stripping of histones), giving a comet-like image with the intensity of the tail depending on the frequency of breaks which relax supercoiling and allow migration of the DNA loops containing the breaks (Cook et al., 1976; Azqueta and Collins, 2013). If nucleoids are digested with lesion-specific endonucleases, different DNA lesions can be detected: formamidopyrimidine DNA glycosylase (FPG) detects oxidized purines, formamidopyrimidines (ring-opened adenine

Table 1 | Overview of human DNA repair systems.

Repair pathway	Damage repaired	Sources of damage
Direct reversal	Alkylated base O^6-methyl-G; pyrimidine dimers (by photolyase)	Alkylating agents, nitrosourease, streptozotocin, UV(C) light
Base excision repair	Oxidized bases, alkylated bases, abasic/apurinic/apyrimidinic sites, single-strand breaks	Reactive oxygen species (ROS), alkylating agents, ionizing radiation, spontaneous hydrolysis
Nucleotide excision repair	Bulky helix-distorting lesions, intra-strand cross links, DNA–protein cross links, inter-strand cross links	UV(C) light, cigarette smoke, dietary factors [aflatoxin, PAHs (benzo[a]pyrene)]
Mismatch repair	Mismatched base pairs, small insertion loops	Replication errors, minor base modifications (oxidation, alkylation)
Double-strand break repair; i.e., homologous recombination and non-homologous end-joining	Double-strand breaks	Ionizing radiation, replication errors

Adapted from Tyson and Mathers (2007).

or guanine) and ring-opened N7 guanine adducts produced by alkylating agents; 8-oxo-guanine (8-oxoG) DNA glycosylase (OGG1) detects oxidized purines and formamidopyrimidines; endonuclease III detects oxidized pyrimidines; T4 endonuclease V detects dimerized pyrimidines (induced by UV); 3-methyladenine DNA glycosylase II (AlkA) detects 3-methyladenine; finally uracil DNA glycosylase (UDG) detects uracil misincorporated in DNA (Azqueta and Collins, 2013). Several enzymes are still under consideration to be combined with the comet assay to detect other DNA lesions.

This assay was used to measure not only DNA lesions but also DNA repair from its very beginning. The first work that refers to this technique was published by Ostling and Johanson (1984) and they studied SBs rejoining in γ-irradiated L5178Y-S cells (a murine lymphoma cell line). They used the neutral version of the comet assay where DNA is not denatured. A few years later, Singh et al. (1988) published the first work using the alkaline comet assay, where the DNA helix is unwound by alkaline treatment, and as a consequence of which ALS are converted to breaks. They also used it to study SBs rejoining in X-irradiated human lymphocytes.

These two papers studied the kinetics of repair by performing the comet assay on DNA-damaged cells at different times after incubation, in what has been called the cellular repair assay or the challenge assay (Au et al., 2010; Collins and Azqueta, 2012). The standard comet assay is used to monitor the capacity of cells to rejoin breaks; but if the aforementioned lesion-specific endonucleases are used, the removal of a particular type of lesions can be assessed. It is important that the induced lesions are as "clean" as possible to give confidence that we are monitoring the repair of a specific lesion. SSBs are easily induced by a brief treatment with H_2O_2 or by irradiation with X- or γ-rays; oxidized purines, mainly 8-oxoG, are induced by treating the cells with the photosensitiser Ro 19-8022 plus visible light; alkylated bases are produced by treating the cells with an alkylating agent such as methyl methanesulfonate (MMS) and dimerized pyrimidines are produced by irradiating the cells with UV(C). An optimal dose of irradiation or concentration of chemicals should be used, avoiding saturation of the DNA repair capacity of the cells or the assay. Rejoining of SSBs is a simple process that can go to completion in less than half an hour, while the repair of DSBs or oxidized bases can take hours; thus precise monitoring is required, with several measurements at suitable intervals (rather than a single measurement of damage remaining at one time point) and the repair capacity expressed as $t_{1/2}$ for removal of damage or initial slope of the curve (Collins and Azqueta, 2012). A different modification of the cellular repair assay is needed to study cross link repair, since in this case the movement of DNA during electrophoresis is blocked by the cross links. Therefore, at each incubation timepoint, cells are treated with an agent such as X-rays to induce breaks before performing the comet assay; repair is indicated by an increase in comet tail intensity as the blockage of X-ray-induced migration is progressively released (Spanswick et al., 2010).

A modified version of the challenge assay, the Comet-FISH assay – a combination of the comet assay with fluorescent *in situ* hybridization (FISH), using labeled probes to particular DNA sequences – has been used to study DNA repair of single genes or DNA sequences (Shaposhnikov et al., 2011). In this assay, the DNA damage repair in a specific gene can be monitored by following the "retreat" of the gene-specific signals from the comet tail to the comet head over time. In addition, the Comet-FISH assay can be used as an alternative to Southern-blotting and ligation-mediated PCR techniques to study transcription-coupled repair (TCR) of physiologically relevant levels of DNA lesions (Spivak et al., 2009; Guo et al., 2013).

Another approach to measuring the DNA repair activity with the comet assay is to measure the accumulation of DNA breaks, as incision events, by blocking repair synthesis. This approach has been used to measure NER, employing inhibitors (aphidicolin, or cytosine arabinoside in combination with hydroxyurea) of the DNA polymerase that participates in this repair pathway (Gedik et al., 1992; Vande Loock et al., 2010).

THE COMET-BASED "*IN VITRO*" DNA REPAIR ASSAY

The above approaches to measure DNA repair activity are not ideal for biomonitoring trials where many samples have to be processed at the same time. For this scene another strategy to measure BER or NER in cells using the alkaline comet assay has been developed (Collins et al., 2001; Langie et al., 2006; Gaivão et al., 2009; van Dyk et al., 2010; Hasplova et al., 2012). It is a biochemical approach, called the comet-based *in vitro* assay, in which DNA nucleoids containing a specific lesion (the substrate; derived by lysis of cells that have been treated with an appropriate damaging agent) are incubated with a cell extract containing a certain amount of repair enzymes (**Figure 1**). These enzymes, as a part of the repair process, induce breaks at the site of the lesions in the substrate that are measured using the alkaline comet assay protocol. The capacity of the cell extract to carry out the incision, considered to be the rate-limiting step of the repair process, is taken as an indicator of the DNA repair activity of those cells. Collins et al. (1994) demonstrated, using an early version of this assay, that the extract is capable of finishing the NER process if deoxyribonucleotides and ATP are provided. The nature of the lesions in the substrate nucleoids defines the repair pathway that it is going to be studied; for example BER can be measured with nucleoids containing 8-oxoG (induced by the photosensitizer Ro 19-8022 plus light) and NER with nucleoids containing dimerized pyrimidines [induced by UV(C)]. Substrate nucleoids should contain an excess of lesions for the extract to work, but unwanted lesions, including breaks, should be low. The time of incubation of the extract with the substrate should also be critically chosen to be able to differentiate levels of repair activity between extracts. It is also crucial to include in a parallel incubation non-damaged substrate nucleoids to determine the action of non-specific nucleases (Azqueta et al., 2009; Gorniak et al., 2013).

The current review will give an overview of the various studies in which the comet-based *in vitro* DNA repair assay has been applied so far, highlighting the most important findings as well as discussing shortcomings. The focus will not be on the practical challenges that might arise when applying the assays, since the sources of potential problems and practical advices have been published recently (Azqueta et al., 2013a; Slyskova et al., 2014c) together with a detailed protocol of this approach to measure BER and NER in cultured cell lines, blood cells, animal tissues, and human biopsies. A comet-based *in vitro* assay for cross link repair has also been developed (Herrera et al., 2009).

STUDIES USING THE COMET-BASED *IN VITRO* DNA REPAIR ASSAY

The comet-based *in vitro* DNA repair assay has been used in some cell culture and animal studies but it is mostly used in human biomonitoring. In this section, we will briefly review the different *in vitro*, *in vivo* animal and human studies where this technique has been applied to measure DNA repair activity.

CELL CULTURE STUDIES

There are very few studies in the literature where the comet-based *in vitro* DNA repair assay has been applied. Silva et al. (2008) published the first paper using this technique to measure BER activity

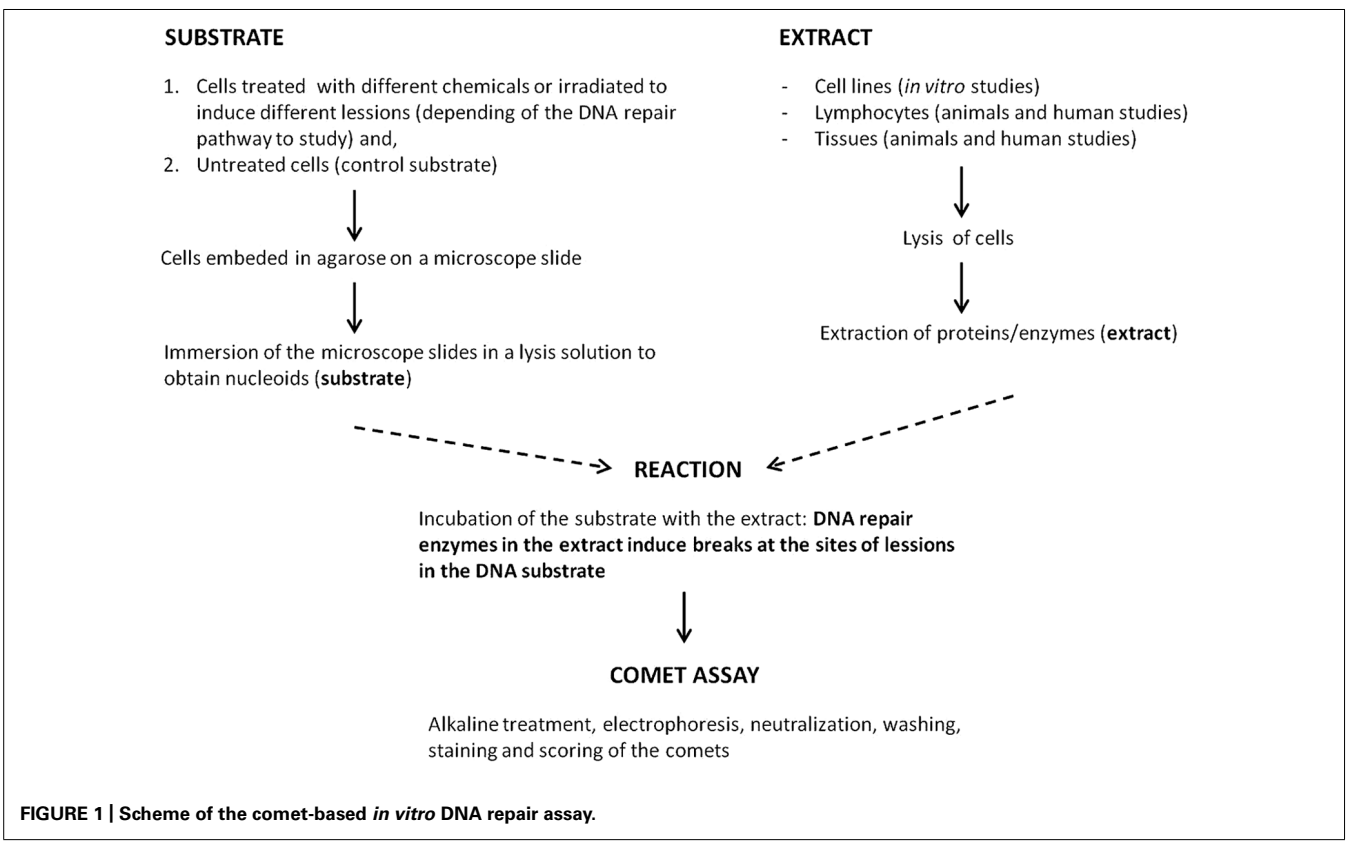

FIGURE 1 | Scheme of the comet-based *in vitro* DNA repair assay.

in cell culture. They studied the effect of different polyphenols on the BER activity of PC12 cell (derived from rat pheochromocytoma) and found a significant increase in the incision activity of extracts from cells treated with rosmarinic acid. A year later they examined two synthetic nitrogen compounds, developed as antioxidant drugs, but they did not find any such effect on repair (Silva et al., 2009). Also Sliwinski et al. (2008) published the first paper using the technique to measure NER activity in cell culture. They measured the effect of ST1571, a drug used in the treatment of chronic myeloid leukemia which inhibits the activity of the BCR/ABL oncogenic kinase, on the NER activity of different human lymphoid leukemia cells. They found that extract from BCR/ABL cells treated with the drug showed a highly significant decrease in incision activity.

Extract from HeLa cells (derived from human cervical cancer) and Caco-2 cells (derived from human colon carcinoma) treated with β-cryptoxanthin showed a significant increase in BER activity compared with non-treated cells (Lorenzo et al., 2009). Incubation of Caco-2 cells with water extracts of Salvia species, luteonil-7-glucoside and rosmarinic acid also increased the BER activity of the cells though it was non-significant for rosmarinic acid (Ramos et al., 2010a). The same group demonstrated a significant increase in the BER activity of extract from Caco-2 cells incubated with ursolic acid but not with luteolin (Ramos et al., 2010b).

Azqueta et al. (2013b) showed that vitamin C caused DNA breaks in nucleoids (substrate) when trying to carry out the comet-based *in vitro* repair assay to study the effect of vitamin C on BER of Caco-2 cells. This finding made it impossible to carry out this test since vitamin C was present in cell extracts and masked the results (Azqueta et al., 2009, 2013b).

The effect of hereditary tyrosinemia type 1 metabolites on DNA repair was studied by van Dyk et al. (2010) in HepG2 cells (derived from human hepatoma). This disorder, caused by a defective fumarylacetoacetate hydrolase enzyme, causes the accumulation of metabolites such as succinylacetone and *p*-hydroxyphenylpyruvate. The authors studied the BER and NER incision activity in extract from cells treated with both metabolites. They used H_2O_2- and methyl methanesulfonate (MMS)-treated cells to produce the nucleoids for studying BER and benzo[a]pyrene-treated cells to produce the nucleoids to study NER. Both metabolites decreased the DNA repair activity of the cells, the effects being more pronounced in BER than in NER.

In some of these studies, there is a lack of proper controls for the correct interpretation of the results. Azqueta et al. (2009) warned that non-treated nucleoids, as substrate, should always be used to allow for the possible presence of non-specific nucleases. They also pointed out the possibility that the test compound might itself directly induce breaks in the nucleoids (substrate) and its presence in the extract thus interfere with the assay.

ANIMAL STUDIES

Although comet-based assays are easy to use, sensitive, versatile, and relatively inexpensive, to the best of our knowledge, there are only a few reports that describe the use of animal tissue extracts in the comet-based assay to measure activities of NER (Langie et al., 2010a) or BER (Mikkelsen et al., 2009; Langie et al., 2011, 2013, 2014; Gorniak et al., 2013) *in vitro*.

Mikkelsen et al. (2009) were the first to apply the *in vitro* repair assay to study BER-related DNA incision activity of protein extracts from lung and liver of aging mice. However, they did not include a control of low-damage nucleoids (e.g., for BER; nucleoids not exposed to the photosensitizer Ro 19-8022 plus light), incubated with protein extract in their assay. Inclusion of these controls is important to detect the possible presence of non-specific nuclease activity, preventing misinterpretation of the findings as has been reported by Langie et al. (2011) and Gorniak et al. (2013) for tissues and by Azqueta et al. (2009) for cultured cells. Moreover, the non-specific nuclease activity can differ markedly between various tissues in the same animal, and so direct comparisons of DNA incision activity in different tissues should be interpreted with caution. Recently, Langie et al. (2011) optimized the comet-based assay for measuring BER-related DNA incision activity in animal tissues, specifically with mouse tissues (Gorniak et al., 2013). The problem of non-specific nuclease activity was overcome by the addition of 1.5 μM aphidicolin in DMSO and selection of a reliable protein concentration, allowing specific detection of DNA repair incision activity. Whether aphidicolin could possibly enhance detection of NER activity by preventing the occurrence of non-specific nuclease activity or any repair synthesis, has not been rigorously tested yet.

So far, the comet-based *in vitro* DNA repair assay has mainly been used to study the effect of aging or nutritional interventions in animal tissues. Our recent studies (Langie et al., 2011; Gorniak et al., 2013) showed significant age-related declines in BER-related DNA incision activity in brain, lung, and colon tissues of rodents, while incision activity was observed to increase with age for liver (Mikkelsen et al., 2009; Langie et al., 2011). In addition, differences in BER-related DNA incision activity were observed between proliferative and non-proliferative tissues (Mikkelsen et al., 2009; Gorniak et al., 2013). Furthermore, dietary restriction has been shown to influence DNA repair, increasing BER activity in liver as compared to *ad libitum* fed animals (Langie et al., 2011). Recently, much effort has gone into studying the effect of prenatal dietary interventions. Langie et al. (2010a, 2014) observed maternal supplementation with micronutrients to enhance NER activity in the colon and BER activity in the hippocampus of piglet offspring. A maternal low-folate diet during pregnancy and lactation was reported to enhance BER-related incision activity in weaning mice but to reduce BER activity once the offspring reached adulthood (Langie et al., 2013).

Although measuring DNA repair in mammalian tissues using the comet-based assay remains a challenge because of the high levels of non-specific activity, the adapted and optimized assay for quantification of BER-associated incision activity in rodent tissues opens opportunities for a wide range of *in vivo* studies on BER including effects of environmental exposures (such as toxins, dietary factors and pharmaceutical agents) and of physiological processes including growth, development, degenerative diseases, and aging.

Drosophila melanogaster is a model organism with practical and theoretical advantages such as its ease of manipulation, its short life cycle, its xenobiotic metabolizing system (Hallström et al., 1984; Søndergaard, 1993), antioxidant enzymes, and DNA repair pathways (Sekelsky et al., 2000) that are similar or equivalent

to those in mammals, and the detailed knowledge of its genome (Adams et al., 2000). It is an established insect model for human diseases and toxicological research, recommended by the European Centre for the Validation of Alternative Methods (ECVAM). Moreover, strains are available that are efficient and deficient for the several repair systems. The comet assay has been successfully applied to *Drosophila* to study not only genotoxicity but also DNA repair.

Very recently, the comet-based *in vitro* repair assay has been applied to *D. melanogaster* to measure the DNA repair activity in extracts from different strains, proficient and deficient in DNA repair, using wild-type neuroblast cells treated *in vivo* with 1 mM MMS as substrate (Gaivão et al., 2014; Rodríguez et al., submitted). This last work demonstrates the feasibility of an *in vitro* approach to *Drosophila* repair, and – by analyzing extracts of different *Drosophila* strains (such as *mus201*, *mus308* and *mus20, mus308*) – shows that genetic differences are reflected in phenotype and can be quantitated. The *in vitro* approach can provide information about the genetic basis and regulation of specific repair enzymes (Rodríguez et al., submitted).

HUMAN STUDIES

Individual DNA repair activity is a valuable biomarker since it has been regarded as a marker of susceptibility to mutation and cancer. A high repair activity is related to a decrease of the chance of unrepaired damage when cells replicate and so to a decrease in potential mutations. On the other hand, a high repair activity can also reflect exposure to DNA-damaging agents which might induce synthesis of the repair enzymes. Anyway a high repair activity is always a good thing but more evidence is needed to confirm that the DNA repair activity is a biomarker of susceptibility to cancer.

The *in vitro* repair assay based on the comet assay has been particularly useful in human trials; samples of cells or tissue, or cell extracts, can be frozen at −80°C for long periods before the repair assay is carried out, which is advantageous when, typically, samples are collected from several subjects on the same occasion, and often other samples have to be collected and other assays performed.

Two studies have applied the *in vitro* assay in order to investigate DNA repair activity against the background of other biomarkers of genotoxicity. Etemadi et al. (2013) aimed to explain variability in PAH-related adducts among non-smokers by evaluating genetic polymorphisms and individual NER activity. In this study, phase I SNPs and NER activity explained 17% of the variation in PAH DNA-adduct levels. The association between oxidative DNA damage, antioxidant serum capacity and BER activity was investigated in healthy non-smokers, but no strong relationships were observed (Tsai et al., 2013).

Though the DNA repair activity is determined genetically, it is also affected by environmental conditions such as nutritional and lifestyle factors. As already pointed out, regulation of DNA repair activity is not simply at the level of transcription, and gene expression is not a reliable guide to enzyme activity, so there is a need for a phenotypic assay. The comet-based *in vitro* DNA repair assay has been used mainly in nutritional intervention studies but also in occupational and clinical studies, as described in the next subsections.

Occupational studies

Every day, human populations are exposed to mutagenic and carcinogenic compounds, both occupational and environmental. In terms of occupational exposure, in several jobs people are exposed to genotoxic/mutagenic compounds, for example: pesticides, hair dyes, formaldehyde, antineoplastic agents, organic solvents, etc.

Dusinska et al. (2004a) measured BER capacity in workers exposed to asbestos, who had significantly higher level of chromosomal aberrations than unexposed factory controls, but no effect of exposure on BER capacity was observed. In another study of the same group, BER capacity was again unaffected by exposure to mineral fibers as measured in workers of rockwool manufacture and compared with administrative employees of the same factory (Dusinska et al., 2004b). Slyskova et al. (2007) measured BER capacity by *in vitro* comet-based assay in styrene-exposed workers as compared to unexposed clerks. Base excision repair capacity did not differ between groups and did not correlate with parameters of styrene exposure or biomarkers of genotoxic effects, namely DNA strand breaks, N1-styrene-adenine DNA adducts, chromosomal aberrations and *HPRT* mutations.

In these studies, while the harmful effect of exposure was clearly recognizable by high levels of various biomarkers of genotoxicity, the effect of exposure on DNA repair activity was not that straightforward or substantial that it could have been observed in relatively small study groups, which is usually the case for occupational studies limited by the number of employees in the factory.

Nutritional studies

Until recently, there was little interest in the regulation of DNA repair by nutritional factors. It was generally assumed that DNA repair is a constitutive, or "housekeeping" function, unlikely to be much affected by exogenous factors. The inter-individual range of repair capacities (both BER and NER) is considerably more than can be explained by differences in genotype; polymorphisms in repair genes have been shown to have little effect on the corresponding enzyme activities. Induction of repair by exposure to DNA-damaging agents is a feasible source of variation, and several researchers have been looking also at the possibility that nutrition plays a role.

The assay was first applied to humans in a trial of coenzyme Q10 in six subjects (Tomasetti et al., 2001). After a week of supplementation with 100 mg Q10 per day, BER activity was almost three times as high as the activity before supplementation. In a crossover designed trial of green kiwifruit (one, two or three per day for 3 weeks, with washout periods between doses), there were highly significant increases in BER activity – without a clear dosage effect (Collins et al., 2003). A later study with golden kiwifruit failed to show any effect on BER or NER (using a substrate of nucleoids from UV(C)-treated cells; Brevik et al., 2011a). An increase in BER was reported with slow-release vitamin C capsules in a 4-week placebo-controlled trial (Guarnieri et al., 2008). There was no significant effect of intervention with a mix of selenium, retinol, β-carotene, vitamin C, and vitamin E for 6 weeks (Caple et al., 2010), nor after a broccoli-rich diet for 10 days in a crossover trial (Riso et al., 2010). A diet rich in fruits and vegetables (600 g/day) resulted in no effect on BER (Guarnieri et al., 2008), whereas a similar study of the effects of antioxidant-rich fruits and vegetables

(Brevik et al., 2011b) showed a significant increase in BER (and a smaller, non-significant increase with three green kiwifruits/day). In the latter study, NER activity was also studied; in this case, repair activity was decreased by both the fruit and vegetable diet and the addition of kiwifruits to the normal diet. This unexpected finding gave rise to the speculation that a lower level of DNA damage resulting from primary protection by phytochemicals led to a failure to induce secondary protection in the form of NER enzymes; in other words, repair activity was not needed as the damage level was low. The NER assay was applied by Langie et al. (2010b) using a substrate containing benzo(a)pyrene diol epoxide-induced bulky adducts; enhanced repair was seen only in subjects carrying multiple low-activity alleles of repair genes. Recently, Slyskova et al. (2014a) analyzed BER and NER capacities in a large cohort of 340 healthy individuals examined for antioxidants intake by food frequency questionnaires and antioxidants plasma levels. They observed that while BER was not associated with antioxidant-rich diet intake, NER was positively correlated with plasma levels of ascorbic acid and α-carotene.

In summary, while it is evident that nutritional factors can influence DNA repair phenotype, results tend to be inconsistent between studies, and further investigations are needed. At present we have no clear indication as to how the modulation of repair is effected; it seems not to be via changes in gene expression (as discussed by Collins et al., 2012).

Clinical studies

In studies investigating DNA repair activity in relation to human diseases, the cellular or challenge assay has commonly been applied; the *in vitro* DNA repair comet-based assay has been used only rarely, on peripheral blood cells of study subjects, but also on tumor samples.

Base excision repair has been assessed in patients suffering chronic renal failure, showing no association between BER activity and duration of hemodialysis (Stoyanova et al., 2014). Slyskova et al. (2012) determined BER and NER activities in tumors from colorectal cancer patients and observed that the activities of both pathways did not differ from those of healthy adjacent tissue. This study however showed the positive correlation of both pathways between peripheral lymphocytes and colon mucosa, supported also by Herrera et al. (2009).

Since *in vitro* DNA repair comet-based assay to study BER and NER in human solid tissues was optimized only recently (Slyskova et al., 2012, 2014b), more clinical studies on DNA repair in relation to tissue-specific diseases might be expected to be released in the near future.

CONCLUDING REMARKS

The *in vitro* comet-based DNA repair assay is simple and versatile. Base excision repair or NER can be measured by using substrate nucleoids with appropriate DNA lesions. The assay is particularly suitable where many samples need to be assessed and compared in a short time, which is the case in human molecular epidemiology studies of occupational exposure, nutrition, lifestyle, aging, etc.

Integration and comparison of results from different laboratories is only possible if standard protocols are adopted. The assay depends critically on the protein concentration in the extract, reflecting the number of cells or amount of tissue used; this should therefore be constant. Validation of the assay against other repair assays is still needed, and a ring-study to compare assay performance in different laboratories should be carried out.

Results to date have demonstrated the range of repair activities in a healthy human population – a range far greater than can be explained by genetic polymorphisms. This emphasizes the importance of regulation of repair by environmental and/or intrinsic factors – about which we still know relatively little.

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Monitoring regulation of DNA repair activities of cultured cells in-gel using the comet assay

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Base excision repair (BER) is the predominant cellular mechanism by which human cells repair DNA base damage, sites of base loss, and DNA single strand breaks of various complexity, that are generated in their thousands in every human cell per day as a consequence of cellular metabolism and exogenous agents, including ionizing radiation. Over the last three decades the comet assay has been employed in scientific research to examine the cellular response to these types of DNA damage in cultured cells, therefore revealing the efficiency and capacity of BER. We have recently pioneered new research demonstrating an important role for post-translational modifications (particularly ubiquitylation) in the regulation of cellular levels of BER proteins, and that subtle changes (∼20–50%) in protein levels following siRNA knockdown of E3 ubiquitin ligases or deubiquitylation enzymes can manifest in significant changes in DNA repair capacity monitored using the comet assay. For example, we have shown that the E3 ubiquitin ligase Mule, the tumor suppressor protein ARF, and the deubiquitylation enzyme USP47 modulate DNA repair by controlling cellular levels of DNA polymerase β, and also that polynucleotide kinase phosphatase levels are controlled by ATM-dependant phosphorylation and Cul4A–DDB1–STRAP-dependent ubiquitylation. In these studies we employed a modification of the comet assay whereby cultured cells, following DNA damage treatment, are embedded in agarose and allowed to repair in-gel prior to lysis and electrophoresis. Whilst this method does have its limitations, it avoids the extensive cell culture-based processing associated with the traditional approach using attached cells and also allows for the examination of much more precise DNA repair kinetics. In this review we will describe, using this modified comet assay, our accumulating evidence that ubiquitylation-dependant regulation of BER proteins has important consequences for overall cellular DNA repair capacity.

Keywords: base excision repair, DNA repair, DNA damage, ubiquitin, comet assay, ubiquitylation, DNA polymerase β, polynucleotide kinase phosphatase

THE BASE EXCISION REPAIR (BER) PATHWAY

The human genome is constantly exposed to agents that cause damage to DNA, for example endogenously through products of cellular oxidative metabolism, and exogenously through environmental agents such as ionizing radiation. These agents can cause damage to the DNA phosphodiester backbone resulting in the formation of DNA strand breaks, attack to DNA bases resulting in oxidation events (e.g., 8-oxoguanine) or even cause loss of the DNA base itself (apurinic/apyrimidinic or AP site). Such events have been estimated to occur at approximately 10,000 per human cell per day (Lindahl, 1993), and if left unrepaired, these types of DNA damage have been implicated in the development of several human disorders, such as in premature aging, in neurodegenerative diseases, and in cancer. Consequently, the base excision repair (BER) pathway has evolved as the major cellular system which is directly involved in the removal and repair of damaged DNA bases, as well as DNA single strand breaks (SSB) that contain various modifications on the 5′- and/or 3′-end (Parsons and Dianov, 2013). BER is therefore a vital DNA repair pathway directly involved in the maintenance of genome stability and consequently contributes to suppressing the development of human diseases.

BER can be divided into several major steps, each of which is performed by a specific enzyme or class of enzymes that recognize and process the DNA damage or DNA damage intermediate. The majority of BER is achieved through the short-patch pathway that involves the removal and replacement of a single damaged base (Dianov et al., 1992; **Figure 1**, central scheme). In the first step, a damage-specific DNA glycosylase excises the damaged base by cleavage of the N-glycosylic bond linking the damaged base to the sugar phosphate backbone. Currently, there are eleven known human DNA glycosylases, each has its own substrate specificity (Jacobs and Schar, 2012). For example, 8-oxoguanine DNA glycosylase (OGG1) is the major DNA glycosylase involved in the excision of 8-oxoguanine residues, whereas endonuclease III homolog (NTH1) excises oxidized pyrimidines, such as thymine glycol, 5-hydroxycytosine, and 5-hydroxyuracil. Once the damaged DNA base is removed, the second step is performed by AP endonuclease-1 (APE1) which incises the phosphodiester- backbone 5′- to the abasic site to create a DNA SSB flanked by 3′-hydroxyl and a 5′-deoxyribosephosphate (dRP)

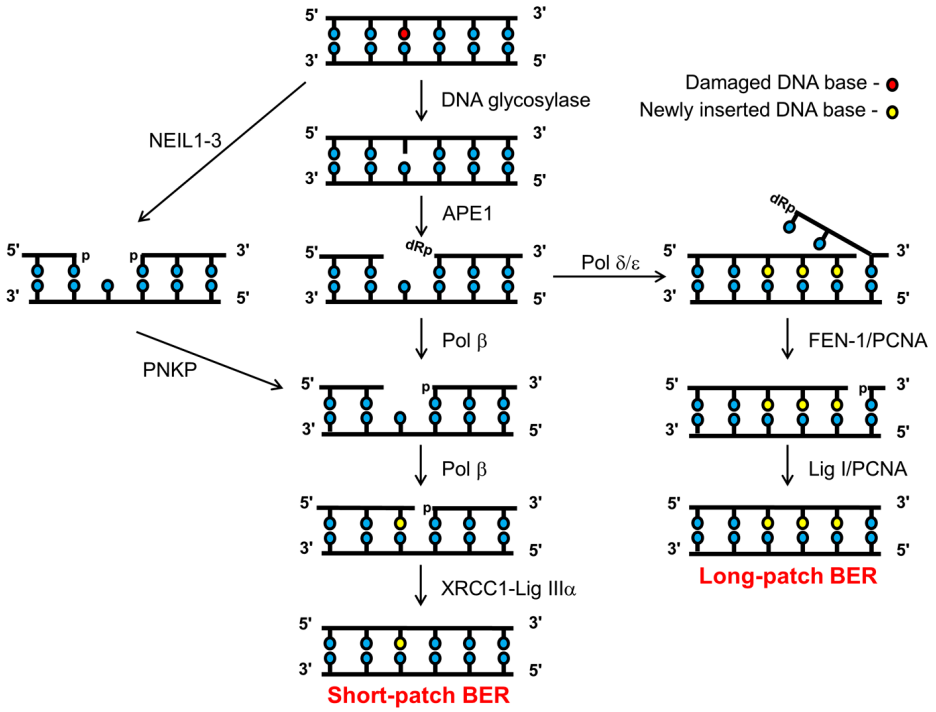

FIGURE 1 | Schematic of the BER pathway. Base excision repair (BER) is initiated by a damage specific DNA glycosylase that excises the damaged base to create an abasic site, which is then incised by APE1 creating a DNA SSB flanked by 3′-hydroxyl and 5′-dRP ends. Pol β cleaves the 5′-dRP moiety and simultaneously adds a single correct nucleotide into the one-nucleotide gap. Finally, the DNA SSB ends are sealed by the XRCC1-Lig IIIα complex, which completes the short-patch BER pathway (central branch). However, if the 5′-dRP moiety is resistant to cleavage by Pol β, then a polymerase switch occurs involving the recruitment of Pol δ/ε which add 2–8 more of the correct nucleotides into the repair gap. Pol δ/ε activity creates a 5′-flap structure that is subsequently excised by FEN-1 in a PCNA-dependent manner. The remaining DNA SSB ends are then sealed by Lig I to complete the long-patch BER pathway (right branch). Alternatively, repair of oxidized DNA bases initiated by the NEIL (1–3) glycosylases, generates a one nucleotide gap flanked by 3′- and 5′-phosphate ends (left branch). The 3′-phosphate is removed by PNKP, generating a substrate amenable for one nucleotide insertion by Pol β and subsequent ligation by XRCC1-Lig IIIα complex.

ends (Demple et al., 1991; Robson and Hickson, 1991). Step three involves removal of the 5′-dRP end carried out by the dRP lyase activity of DNA polymerase β (Pol β), which also simultaneously inserts the complementary nucleotide into the DNA repair gap thus generated (Dianov et al., 1992; Matsumoto and Kim, 1995; Sobol et al., 1996). In the final step DNA ligase IIIα, which is in a complex with X-ray cross complementing protein-1 (XRCC1), then seals the remaining nick in the DNA backbone to restore genome integrity (Cappelli et al., 1997; Nash et al., 1997). The pathway described above is employed, in the main, for the repair of >80% of damaged DNA bases and is commonly referred to as the short-patch BER pathway (Dianov and Parsons, 2007). In instances (within step 3) where the 5′-end is resistant to the dRP lyase activity of Pol β, then there is a polymerase switch to the replicative DNA polymerases, Pol δ/ε. These DNA polymerases add several (2–8) nucleotides into the repair gap, thus creating a 5′-flap structure (**Figure 1**, right scheme). This flap structure is recognized and excised by flap endonuclease-1 (FEN-1), in association with the processivity factor proliferating cell nuclear antigen (PCNA). Finally DNA ligase I (Lig I) then seals the remaining nick in the DNA backbone to complete the long patch BER pathway (Frosina et al., 1996; Podlutsky et al., 2001).

Over the last 10 years, a further sub-pathway of BER has been uncovered through the discovery of the endonuclease VIII-like (NEIL) DNA glycosylases (**Figure 1**, left scheme). Rather than generating an AP site for APE1 activity, the enzymes (NEIL1, 2, and 3) excise the damaged base creating a DNA SSB flanked with 5′- and 3′-phosphate ends (Hazra et al., 2002a,b; Takao et al., 2002; Liu et al., 2010). The 3′-phosphate subsequently requires removal by polynucleotide kinase phosphatase (PNKP), which then creates the 3′-hydroxyl end that is required for Pol β activity (Wiederhold et al., 2004). Following nucleotide insertion, the nick is finally sealed by DNA ligase IIIα-XRCC1, as per the short-patch BER pathway. The NEIL glycosylases appear to have a similar substrate specificity to the major oxidative DNA glycosylases, OGG1 and NTH1, in that they recognize oxidized purines and pyrimidines. However, it is currently unknown what proportion of these oxidized DNA bases are repaired through the NEIL-dependant pathway. Intriguingly, there is some suggestion that these enzymes have a preference for specific, novel oxidative DNA damage, or that they have a preference for single stranded DNA or DNA bubble structures that may be generated through DNA replication (Dou et al., 2003; Hailer et al., 2005; Parsons et al., 2005, 2007; Chan et al., 2009; Zhou et al., 2013).

REGULATION OF BER THROUGH THE UBIQUITIN PROTEASOME PATHWAY (UPP)

BER proteins, particularly over the last decade, have been discovered to be subject to post-translational modifications, such as phosphorylation, acetylation, and ubiquitylation, that have been shown to regulate protein activity, cellular localization, protein–protein interactions, as well as protein stability (Almeida and Sobol, 2007). Recently, there has been accumulating interest in ubiquitylation mediated through the ubiquitin proteasome pathway (UPP), as a means of regulating BER proteins, particularly the cellular steady state levels of BER proteins but also those in response to oxidative stress (Dianov et al., 2011; Parsons and Dianov, 2013). This is particularly important since BER misregulation leading to altered enzymes levels has been frequently observed in several human disorders, such as in premature aging, in cancer, and in neurodegenerative diseases (Coppede and Migliore, 2010; Wilson et al., 2011; Wallace et al., 2012). This evidence highlights a critical role for regulating BER capacity in the maintenance of genome stability and in human disease prevention. Interestingly, BER protein levels do not change dramatically in the cellular response to acute DNA damage induced by exogenous mutagens, suggesting that mammalian cells have a limited capacity to be able to repair the ensuing DNA damage. This suggests that DNA damage repair is achieved by multiple repair cycles of available BER enzymes although if the repair capacity of the cell is exceeded for a significant length of time, then the cell may undergo apoptosis thus avoiding the accumulation of genetic alterations. There are now data emerging to suggest that the cellular steady-state levels of BER enzymes, and therefore the corresponding DNA repair capacity, are adjusted to the cellular levels of DNA damage so that the rate of generation of DNA lesions is comparable to the rate of their immediate repair (Parsons et al., 2008). Indeed, the UPP has been discovered to play a vital role in modulating the BER capacity of the cell and adjusting it to the cellular levels of endogenous DNA damage.

The UPP involves adding the ubiquitin moiety (76 amino acids, 8 kDa protein) onto specific lysine residues on the target protein, and is performed by a cascade of enzymes (**Figure 2**; Weissman et al., 2011). The UPP is initiated by an E1 activating enzyme which forms a thioester with the ubiquitin molecule. The activated ubiquitin molecule is then transferred to a ubiquitin conjugating enzyme (E2) that complexes with an E3 ubiquitin ligase and the target protein, which is then modified with the ubiquitin moiety on particular lysine residues. The specificity of the pathway is achieved at the level of the E3 ubiquitin ligases, since these bind and target specific proteins for ubiquitin attachment. Indeed, >500 E3 ubiquitin ligases are thought to exist in human cells and these can be classified into HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene) and U-Box domain containing enzymes. When a target protein is modified with a single ubiquitin molecule (termed monoubiquitylation), this usually is involved in regulating protein activity, cellular localization or protein–protein interactions. In contrast, the addition of branched ubiquitin chains, which are formed through internal lysines on the ubiquitin molecule (i.e., K6, K11, K27, K29, K33, K48, and K63), also termed polyubiquitylation, usually targets the protein to the proteasome (particularly K48 linkages) where it is subsequently degraded.

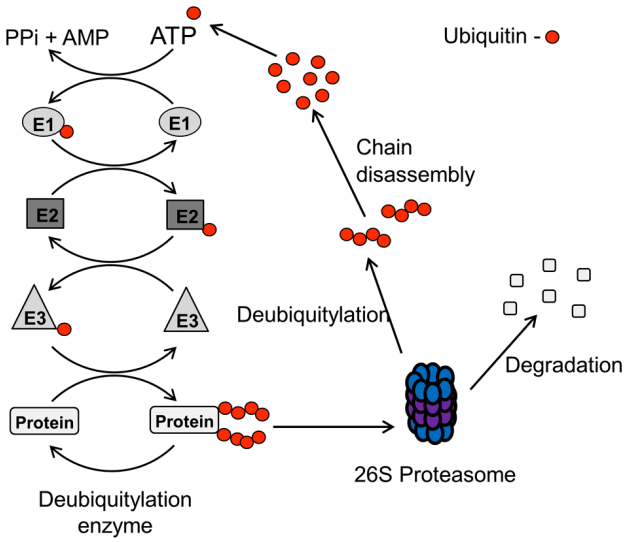

FIGURE 2 | Schematic of the UPP pathway. The UPP pathway is initiated by an E1 activating enzyme in an ATP-dependant process which forms a thioester with the ubiquitin molecule. The activated ubiquitin molecule is then transferred to a ubiquitin conjugating enzyme (E2), and the E2-ubiquitin complex then binds with an E3 ubiquitin ligase and the target protein. The E3 ubiquitin ligase transfers the ubiquitin moiety onto specific lysine residues within the target protein, and the formation of ubiquitin chains through internal lysine residues within the ubiquitin protein (termed polyubiquitylation), usually targets the protein for degradation by the 26S proteasome. Protein degradation is achieved by deubiquitylation, and subsequent disassembly, of the ubiquitin chains from the ubiquitylated protein which is then degraded, and the ubiquitin protein is recycled.

In addition to E3 ubiquitin ligases, enzymes that are able to reverse the effects of ubiquitylation exist, which are known as deubiquitylation enzymes. Approximately 90 deubiquitylation enzymes have been shown to be present in human cells that are able to hydrolyse ubiquitin chains, and they consist of five families of enzymes. These are the ubiquitin specific proteases (USPs), ubiquitin COOH-terminal hydrolases (UCHs), ovarian tumor proteases, Josephins, and the JAB1/MPN/MOV34 (JAMMs) family (Clague et al., 2013). Deubiquitylation enzymes also appear to demonstrate a degree of substrate specificity, and therefore play a critical role in the regulation of the levels of key cellular proteins.

Only recently has the UPP been shown to play an important role in BER regulation by modulating cellular levels of key BER proteins (Parsons and Dianov, 2013). Indeed, both monoubiquitylation and polyubiquitylation of BER proteins has been discovered. Monoubiquitylation has been shown to regulate cellular localization and/or protein activity, as well as being a precursor for subsequent polyubiquitylation that usually targets the protein for proteasomal degradation. Consequently, these cellular mechanisms play important roles in the response to acute DNA damage, in which BER proteins levels can marginally increase to accommodate a small increase in DNA damage load. This suggests that the levels of BER proteins are finely tuned to the

amount of endogenous DNA damage. There is also emerging evidence suggesting considerable crosstalk between BER protein ubiquitylation and other post-translational modifications, such as phosphorylation, which have been shown to control BER protein levels via modulating ubiquitylation-dependant degradation.

MEASURING DNA REPAIR IN-GEL USING THE COMET ASSAY

The comet assay, also known as the single cell gel electrophoresis assay, is a very sensitive and rapid quantitative technique used to detect DNA damage at the individual cell level. Originally developed in 1984 by two Swedish scientists, Ostling and Johanson (1984), it has since become widely used as a technique for the evaluation of DNA damage in a wide variety of cell types. The assay allows for visual evidence of DNA damage in a eukaryotic cell to be measured, based on the quantification of DNA containing breaks migrating from the cell nucleus during electrophoresis, to generate the characteristic "comet" images following DNA staining and image analysis. The most widely performed version is the alkaline comet assay (Singh et al., 1988), since the DNA unwinding and electrophoresis steps are performed in high alkaline buffer, which reveals the presence of alkali-labile sites (AP sites), in addition to DNA double strand breaks and DNA SSBs. As well as measuring DNA damage, the assay can also be employed at various time points post-treatment of cells, to measure the rate of DNA damage repair. Indeed, the comet assay is widely used to measure cellular DNA repair capacity, and also to monitor changes in this repair capacity in repair-deficient cells, or in cells that have been genetically manipulated. For example, XRCC1-deficient cells which are unable to perform short-patch BER, have been found to exhibit lower DNA repair rates by the comet assay (Taylor et al., 2000, 2002).

Practically, the assay to measure DNA damage repair involves several steps; treatment of the cells with a DNA damaging agent, a period of incubation time to allow for DNA repair, embedding of the cells in agarose, cell lysis, DNA unwinding, DNA migration by electrophoresis, and finally staining of the DNA for image analysis. Traditionally, a confluent monolayer of cells in tissue culture flasks or dishes is treated with a DNA damaging agent. Following treatment, the cells are washed with buffer (e.g., phosphate buffered saline) to remove the majority of the genotoxin, and then fresh culture medium added to the cells prior to incubation at 37°C in a CO_2 incubator to allow for DNA repair. The attached cells are then removed from the flasks/dishes by trypsinization involving a further incubation at 37°C. Cells are subsequently counted, diluted to the appropriate cell density and mixed with molten agarose (at ∼35°C) prior to embedding onto a microscope slide using a glass coverslip. The agarose is then allowed to set by placing the slides on ice and the coverslip removed prior to immersion of the slide containing the agarose embedded cells in lysis buffer. In combination with this approach, the use of recombinant DNA repair proteins, such as Fpg and Nth1 that recognize oxidized purine and pyrimidines, respectively, can be used to convert oxidized DNA bases to DNA strand breaks and therefore reveal the true extent of the DNA damage and its subsequent repair (Azqueta and Collins, 2013). Whilst this method has the advantage of allowing all the cells to be exposed to a DNA damaging agent at once, thus eliminating variation in exposure levels, the use of trypsin to detach the cells requires caution. If the amount, and incubation time, with trypsin is not correctly controlled, this can increase the basal level of DNA strand breaks therefore causing variation in the amount of DNA damage quantified. Furthermore, this method is extremely laborious and time-consuming, considering the multiple time points (i.e., >5) that require analysis (involving separate cell culture flasks/dishes that necessitate trypsinization and processing). This approach also does not allow the entirely accurate measurement of DNA repair kinetics post-treatment due to the extended cell manipulations prior to embedding in agarose.

As an alternative to the traditional approach (treatment of cultured cells as a monolayer with a genotoxin, followed by incubation prior to cell trypsinization) for measuring DNA repair using the comet assay as described above, variations of this method have been described. For example, one study irradiated cells already embedded in agarose, and the slides were consequently placed in medium to allow for DNA repair prior to cell lysis (Alapetite et al., 1999). We have also more recently described another variation of this method by treating cells in a suspension of medium with a genotoxin, embedding the cells within an agarose matrix and then allowing the cells to repair the DNA damage *in situ* in an humidified chamber (Parsons and Elder, 2003; Woodhouse et al., 2008). In our opinion, this method is less laborious and time-consuming, and can allow the measurement of DNA damage at more precise time points post-treatment. We will therefore detail the major steps involved in this in-gel DNA repair alkaline comet assay (**Figure 3**).

Following trypsinization of actively dividing cells, the cells are counted and diluted accordingly in cell culture medium (∼2×10^5 cells/ml). The cells are then aliquoted (250 μl/well) into the wells of a 24-well plate which is placed on ice to prevent cell adhesion. The cells can then be treated in suspension on ice with the DNA damaging agent, and in particular we have previously used either ionizing radiation or hydrogen peroxide, due to its relatively short half-life in solution. Following DNA damage treatment, the cells are mixed with molten (at ∼35°C) low melting point agarose (1 ml of 1% agarose in PBS) and immediately the agarose/cell suspension (1 ml) is removed. The suspension is added to a microscope slide (76 mm × 26 mm), which had already been precoated with normal melting point agarose (1 ml of 1% agarose in water) and allowed to dry. The cell/agarose mix is covered with a glass coverslip (22 mm × 50 mm) and the slide transferred to a metal tray, on ice, to stimulate the agarose to set. After 2–3 min on ice, the slides can then be transferred to a humidified chamber prewarmed at 37°C (slide box containing damp tissue to create a humid environment), and the cells allowed to undergo DNA damage repair for the appropriate times (i.e., 5–120 min). Following incubation, the slides are removed from the humidified chamber, the coverslip removed and the slides placed in cell lysis buffer containing high salt, detergent, and DMSO [10 mM Tris (pH 10.5), 2.5 M NaCl, 100 mM EDTA, plus 1% DMSO, and 1% Triton X-100; prepared just before use] at 4°C. This step will halt the DNA repair reaction

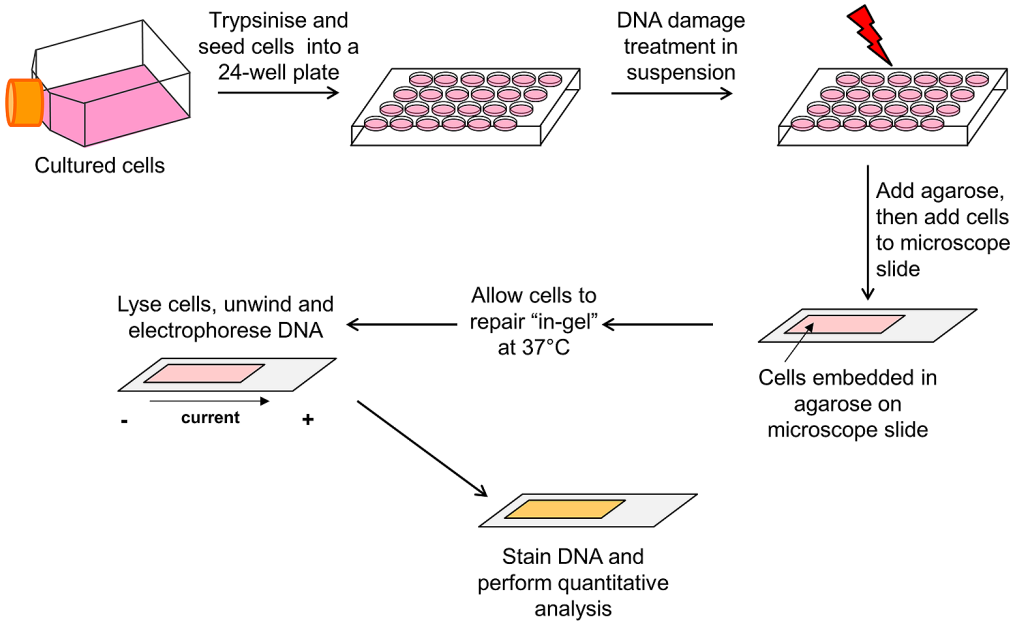

FIGURE 3 | Schematic of the in-gel DNA repair comet assay. Actively dividing cells cultured as a monolayer in flasks/dishes are trypsinized, counted, and diluted in cell culture medium (~2 × 10^5 cells/ml). Cells (250 μl) are aliquoted into the wells of a 24-well plate placed on ice, and then treated in suspension with the DNA damaging agent. Molten low melting point agarose (1 ml) is added to the cells, mixed, and the agarose/cell suspension immediately added to a microscope slide precoated with normal melting point agarose. A glass coverslip is added and the slide placed on ice to allow the agarose to set. The slides are then transferred to a humidified chamber prewarmed at 37°C and the cells allowed to undergo DNA damage repair for the appropriate times. Following incubation, the glass coverslip is removed and the slides placed in cell lysis buffer for at least an hour at 4°C. Following cell lysis, the slides are transferred to an electrophoresis tank containing DNA unwinding/electrophoresis buffer for 30 min to allow the DNA to unwind, and the DNA electrophoresed (25 V, 300 mA for 25 min) to allow DNA migration. The slides are washed with neutralization buffer, allowed to dry overnight and the following day, the agarose is rehydrated and the DNA stained. The slides are allowed to dry again, prior to subsequent quantitative analysis of DNA strand break and alkali labile site measurement using the appropriate image analysis software.

as the cell lysis buffer destroys all cellular membranes and constituents, leaving the DNA intact. Cells are subsequently allowed to lyse for at least an hour (overnight is also possible), and then placed in an electrophoresis tank (darkened thus avoiding light exposure and prevent additional DNA damage induction) and covered with DNA unwinding/electrophoresis buffer (300 mM NaOH, 1 mM EDTA, and 1% DMSO prepared just before use) for 30 min to allow the DNA to unwind. The DNA is electrophoresed at 25 V for 25 min (at 300 mA) to allow the DNA to migrate, after which the slides are covered with neutralization buffer (500 mM Tris–HCl, pH 8.0) for 3 × 5 min washes and the agarose then allowed to dry overnight. The following day, the agarose slides are rehydrated in water (pH 8.0) for 30 min prior to staining (we routinely use 1 ml SYBR Gold diluted at 1:10,000 for 30 min). The slides are allowed to dry again, prior to subsequent analysis (i.e., 50 cells per slide and >2 slides per time treatment; analysis performed using Komet 5.5 from Andor Technology, Belfast, UK). The slides can be stored indefinitely in a dry box in the dark, and rehydrated and restained, if necessary. The in-gel DNA repair comet assay described should be repeated in at least three independent experiments, to ensure reproducibility.

It should be noted that whilst this modified comet assay has its advantages (i.e., less laborious and time-consuming, thus avoiding extensive cell culture based-processing, and allowing more precise DNA damage repair kinetics especially at earlier time points), there is the limitation that any DNA damaging agent with a significantly long half-life in solution cannot be used for treating the cells in suspension. This is since the agent will remain in contact with the cells and continue to damage the DNA during the DNA repair time course period.

MONITORING BER REGULATION USING THE IN-GEL DNA REPAIR COMET ASSAY

We have successfully used the modified alkaline comet assay described above, whereby cells treated in suspension with a DNA damaging agent are embedded in agarose and subsequently allowed to repair in-gel prior to lysis and electrophoresis, to study DNA damage repair kinetics in response to oxidative stress. Specifically, we have most recently employed this technique to strengthen our accumulating evidence that regulation of BER protein levels through the UPP has important consequences for cellular DNA repair capacity, particularly in response to exogenous stress. Principally, we have focussed on the regulation of the cellular levels of the major DNA polymerase employed in BER, Pol β, through the UPP. However, we have also recently examined the cellular mechanism of regulation of PNKP protein levels by the UPP. We will therefore summarize the key important findings of these studies, including how the in-gel DNA repair comet assay has provided vital information on DNA

damage repair kinetics through BER protein modulation. We will also briefly summarize key evidence to date, highlighting an important role for the UPP in the regulation of other BER enzymes.

REGULATION OF POL β PROTEIN LEVELS

Among the BER proteins, Pol β has a very important role in filling the one nucleotide gap that arises during the BER process. The regulation of cellular Pol β protein levels is vital as haploinsufficiency, resulting in reduced BER capacity, has been shown to increase aging and the susceptibility to human diseases, such as cancer (Cabelof et al., 2006; Patterson and Cabelof, 2012). Furthermore, Pol β overexpression has been shown to cause a mutator phenotype in cells (Canitrot et al., 1998) and also Pol β has shown to be overexpressed in approximately 30% of all human cancers (Albertella et al., 2005). Only over the last 5–6 years have we begun to understand the mechanism of regulation of cellular Pol β protein levels, and indeed the crucial role for the UPP in this process. Firstly, in pioneering work, we demonstrated that Pol β is stabilized on damaged DNA in a complex with DNA ligase IIIα-XRCC1 and therefore Pol β protein levels are controlled by the level of endogenous DNA damage (Parsons et al., 2008). In this study we showed that Pol β protein not involved in a complex is targeted for ubiquitylation-dependant proteasomal degradation, as evidenced by decreased Pol β protein levels in XRCC1-deficient cells. The E3 ubiquitin ligase involved in polyubiquitylation of Pol β was discovered using an *in vitro* ubiquitylation assay incorporating Pol β as a substrate in combination with fractionated cell extracts, and was identified as C-terminus of Hsc70 interacting protein (CHIP). The role of CHIP in modulating the steady state levels of Pol β was confirmed as observed by increased Pol β levels in HeLa cells following CHIP siRNA-mediated knockdown.

As a consequence of this study, we discovered an additional level of regulation, whereby Pol β is monoubiquitylated on the same lysine residues (41, 61, and 81) by the E3 ubiquitin ligase Mule (ARF-BP1/HectH9). This monoubiquitylation of Pol β (approximate 20% of the total protein levels) occurs in the cytoplasmic portion of the cell where Mule is predominantly located, prior to subsequent polyubiquitylation-dependent degradation by CHIP (Parsons et al., 2009). Indeed, a knockdown of Mule by siRNA in HeLa or WI-38 cells led to an increase in the cellular protein levels of Pol β, interestingly in both the cytoplasm and nucleus of the cell. Consequently, we used the in-gel DNA repair alkaline comet assay to demonstrate that as a result of increased Pol β following Mule siRNA targeted knockdown, this led to accelerated DNA damage repair rates following oxidative stress. Specifically, when HeLa (**Figure 4A**) or WI-38 cells (**Figure 4B**) were treated with hydrogen peroxide, to induce DNA damage formation, the levels of DNA strand breaks and alkali labile sites discovered by the comet assay were found to be equal in both the presence (red bars) or absence (blue bars) of Mule siRNA (see time 0). However, even at early DNA repair time points (15 min post-treatment), the levels of DNA damage were significantly reduced in the absence of Mule, compared to mock siRNA treated cells. This demonstrates more efficient DNA repair kinetics of hydrogen peroxide induced DNA

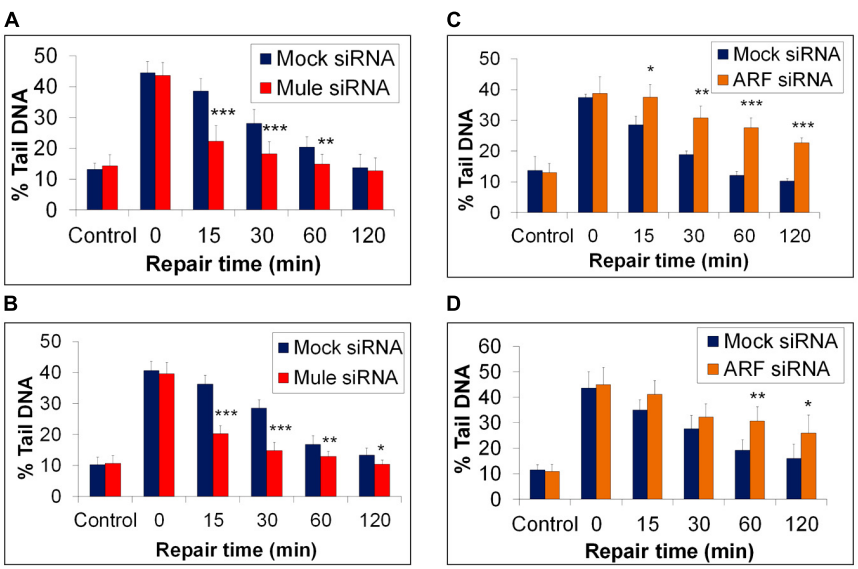

FIGURE 4 | Modulation of BER through Mule-dependent regulation of Pol β protein levels, as revealed by the in-gel DNA repair comet assay. HeLa cells **(A,C)** or WI-38 cells **(B,D)** were treated with Lipofectamine transfection reagent (Life Technologies, Paisley, UK) in the absence (Mock siRNA) and presence of Mule siRNA **(A,B)** or ARF siRNA **(C,D)** for 72 h. Cells were analyzed using the in-gel DNA repair comet assay following treatment in suspension with 20 μM hydrogen peroxide for 5 min and allowing for DNA damage repair at 37°C for up to 120 min. The mean % tail DNA values with SDs from at least three independent experiments were determined using the Komet 5.5 image analysis software (Andor Technology, Belfast, UK). Statistically significant results comparing Lipofectamine and siRNA-treated cells are represented by $*p < 0.02$, $**p < 0.005$, and $***p < 0.001$, as analyzed by Student's *t*-test. Data taken and modified from Parsons et al. (2009).

damage when the Mule protein was absent. Both Mule-proficient and Mule-deficient cells were eventually able to repair all the DNA strand breaks and alkali labile sites initially induced, within the 2 h repair time period, although Mule-deficient cells were able to achieve this within a much shorter time frame (approximately 30–60 min). The observed increased DNA repair rate in cells in the absence of Mule, is consistent with the hypothesis that this is as a direct consequence of increased cellular Pol β protein levels. Since the ARF tumor suppressor protein had previously been discovered to bind and inhibit the E3 ubiquitin ligase activity of Mule (Chen et al., 2005), we conversely depleted ARF using siRNA, which we showed led to an increase in the levels of monoubiquitylated Pol β at the expense of the native protein (Parsons et al., 2009). As a consequence of an siRNA-mediated knockdown of ARF, we again were able to demonstrate using the in-gel DNA repair alkaline comet assay, that DNA repair rates of hydrogen peroxide-induced DNA damage were significantly altered in HeLa (**Figure 4C**) or WI-38 cells (**Figure 4D**). Therefore whilst mock siRNA treated cells (blue bars) demonstrated a complete reduction in the levels of DNA strand breaks and alkali labile sites visualized by the comet assay within 60–120 min post-treatment, cells in the absence of ARF (orange bars) displayed reduced DNA damage repair kinetics. In particular, significantly increased levels of DNA strand breaks and alkali labile sites were still present in ARF-depleted cells 60–120 min post-treatment. This is consistent with the observed reduced levels of Pol β in these cells that is unable to support efficient DNA damage repair rates. However, to support the hypothesis that Mule and ARF regulation of Pol β was directly involved in the modulation of the kinetics of DNA damage repair following hydrogen peroxide treatment, Mule knockdown experiments were performed in Pol β-proficient (Pol $\beta^{+/+}$) and Pol β-deficient (Pol $\beta^{-/-}$) cells. Therefore, in combination with the in-gel DNA repair alkaline comet assay, we revealed that the accelerated DNA repair rates of hydrogen peroxide induced DNA damage observed in HeLa and WI-38 cells following Mule siRNA, could be replicated in Pol $\beta^{+/+}$ cells, as demonstrated by significantly reduced levels of DNA strand breaks and alkali labile sites at early time-points (15 and 30 min) post-treatment (Parsons et al., 2009). In contrast, DNA damage repair rates in Pol $\beta^{-/-}$ cells in the absence and presence of Mule were not significantly different throughout the repair time period, suggesting the dependence of Pol β in DNA repair modulation by Mule.

Whilst we had uncovered roles for the E3 ubiquitin ligases CHIP and Mule, and the ARF tumor suppressor protein in the regulation of the steady state Pol β protein levels, it was unclear how this mechanism could efficiently change in response to changes in the DNA damage environment. It was predicted that a deubiquitylation enzyme may exist that is able to rapidly reverse the effects of mono- and polyubiquitylation of Pol β, and therefore generate more active protein that is required for DNA damage repair. Similar to the studies described above, we used fractionated cell extracts but this time in combination with an *in vitro* deubiquitylation assay incorporating monoubiquitylated Pol β as a substrate, to purify and identify the major Pol β-dependent deubiquitylation enzyme (Parsons et al., 2011). This enzyme was revealed as USP47, a predominantly cytoplasmic protein. Intriguingly, we discovered that USP47 was able to deubiquitylate both mono- and polyubiquitylated forms of Pol β *in vitro*. Following a knockdown of USP47 by siRNA, we observed decreased levels of Pol β in the cytoplasmic compartment of HeLa cells, at the expense of an increase in the monoubiquitylated form of the protein. Whilst an elevation in Pol β protein levels in the nucleus following exogenous DNA damage treatment had previously been observed under normal conditions (Parsons et al., 2008), this was prevented following USP47 siRNA knockdown, suggesting that these cells may be deficient in DNA repair. Therefore, we used the in-gel DNA repair alkaline comet assay to analyze DNA damage repair kinetics of cells in the presence and absence of USP47. We demonstrated that, intriguingly, the levels of DNA strand breaks and alkali labile sites were significantly elevated in HeLa cells deficient in USP47 (**Figure 5A**; green bar) compared to mock siRNA-treated control cells (**Figure 5A**; blue bar) immediately following treatment of the cells with hydrogen peroxide (see time 0). We were also able to show that the repair of this hydrogen peroxide-induced DNA damage was defective throughout the repair time course (15–120 min) in USP47 knockdown cells compared to control cells (**Figure 5A**). In fact, significant levels of DNA damage were still observed 120 min post-treatment with hydrogen peroxide in USP47-deficient cells, highlighting reduced DNA damage repair rates. In addition to this, we now show that cells transfected with USP47 siRNA are also deficient in the repair of DNA damage induced by ionizing radiation (**Figure 5B**; unpublished data). Immediately following ionizing radiation treatment, the levels of DNA strand breaks and alkali labile sites visualized by the in-gel DNA repair alkaline comet assay were similar in Mock siRNA (blue bar) and USP47 siRNA treated cells (see time 0). In contrast following DNA repair, elevated levels of this DNA damage was specifically observed in the USP47 deficient cells at 15–120 min post-irradiation compared to control cells. Cumulatively, these data highlight the importance of USP47, and indeed the regulation of Pol β protein levels, in coordinating an efficient DNA damage repair response.

In summary, these studies have demonstrated important roles for the E3 ubiquitin ligase CHIP and Mule, the tumor suppressor ARF, and the deubiquitylation enzyme USP47 as the major enzymes of the UPP involved in controlling the steady state, and DNA damage-induced, levels of Pol β. This is achieved by controlling the stability of newly synthesized cytoplasmic Pol β, which is used as a source for nuclear Pol β required for DNA damage repair. The in-gel DNA repair alkaline comet assay employed in these studies has been instrumental in examining precise repair kinetics of DNA damage induced by oxidative stress. This method has also been key in improving our understanding of the importance of Pol β regulation in this process, by measuring DNA damage repair rates following modulation of UPP associated enzymes.

REGULATION OF PNKP PROTEIN LEVELS

BER of oxidative DNA base damage that is specifically initiated by the NEIL DNA glycosylases (NEIL1-3) generates a single nucleotide gap flanked by 5′-phosphate and 3′-phosphate DNA ends (**Figure 1**; left branch). Since the 3′-phosphate is not amenable to Pol β activity, through the insertion of the corrected undamaged nucleotide, this requires removal by the

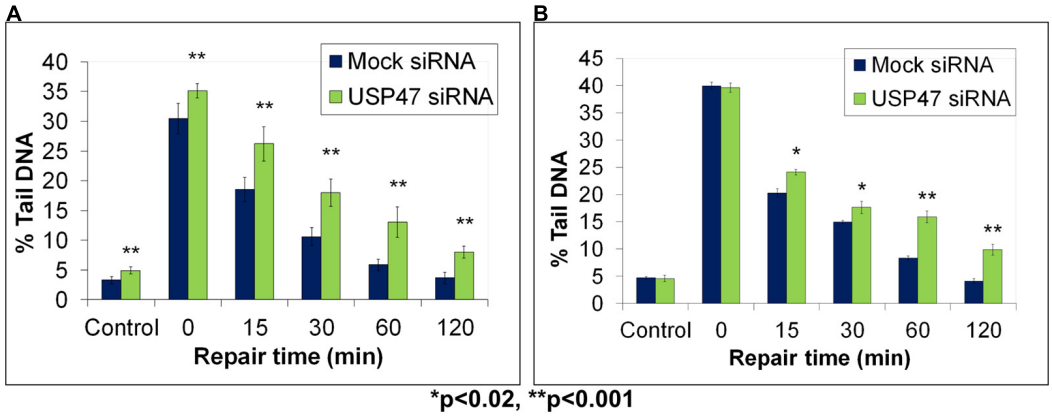

FIGURE 5 | Modulation of BER through USP47-dependent regulation of Pol β protein levels, as revealed by the in-gel DNA repair comet assay. HeLa cells were treated in the absence (Mock siRNA) and presence of USP47 siRNA for 72 h. Cells were then analyzed using the in-gel DNA repair comet assay following treatment in suspension with **(A)** 20 μM hydrogen peroxide for 5 min or **(B)** 8 Gy ionizing radiation and allowing for DNA damage repair. Mean % tail DNA values with SDs were calculated. Further details are provided in **Figure 4** legend. Statistically significant results comparing Lipofectamine and siRNA-treated cells are represented by $*p < 0.02$ and $**p < 0.001$, as analyzed by Student's *t*-test. Panel **(A)** taken and modified from Parsons et al. (2011) and Panel **(B)** represents unpublished data.

3′-phosphatase activity of PNKP (Wiederhold et al., 2004). The importance of PNKP in the cellular DNA damage response has been demonstrated by the observation that an shRNA knockdown of PNKP caused an elevation in the sensitivity of A549 human lung adenocarcinoma cells to oxidative stress induced by hydrogen peroxide and ionizing radiation (Rasouli-Nia et al., 2004). Furthermore, reduced PNKP protein levels caused by a *pnkp* gene mutation have been found in patients suffering from a disease associated with severe neurological abnormalities, termed microcephaly, early-onset, intractable seizures and developmental delay (MCSZ), and lymphoblasts from these patients were found to be defective in the repair of oxidative DNA damage (Shen et al., 2010). These studies have therefore demonstrated the importance of regulating PNKP protein levels in the cellular response to oxidative stress, and in the prevention of human disease. We recently investigated the cellular mechanism of regulation of PNKP protein levels, and discovered that this mechanism involves a cross-talk between phosphorylation and ubiquitylation of the protein, which modifies its ability to be degraded by the proteasome. Whilst ATM-dependant phosphorylation of PNKP on serines 114 and 126 had previously been shown to occur in response to ionizing radiation by two separate studies (Segal-Raz et al., 2011; Zolner et al., 2011), PNKP phosphorylation was suggested to be required for DNA double strand break repair. However, we also discovered that this site-specific PNKP phosphorylation mediated by ATM was also induced following oxidative stress induced by hydrogen peroxide treatment in HCT116p53$^{+/+}$ colorectal carcinoma cells (Parsons et al., 2012). This DNA damage-dependent induction in PNKP phosphorylation was associated with an accumulation of the protein (approximately 50% increase in protein levels), which was found to be mediated through inhibition of ubiquitylation-dependant proteasomal degradation of PNKP (on lysines 414, 417, and 484 within PNKP) catalyzed by the Cul4A–DDB1–STRAP E3 ubiquitin ligase complex. To demonstrate that this cellular mechanism for PNKP regulation has an impact on DNA damage repair, we used the in-gel DNA repair comet assay to measure the kinetics of repair of DNA damage induced by oxidative stress, in the presence and absence of ATM which controls cellular PNKP protein levels (**Figure 6**). Following a knockdown of ATM using siRNA in HCT116p53$^{+/+}$ cells, we showed that these cells (purple bars) have equivalent levels of DNA strand breaks and alkali labile sites generated immediately followed hydrogen peroxide treatment (see time 0) in comparison to mock siRNA treated control cells (dark blue bars). However, following a time course of incubation of cells post-treatment, ATM knockdown cells show an elevation in the levels of DNA damage, specifically between 10 and 60 min post-treatment compared to mock-treated cells, demonstrating that ATM-depleted cells have a DNA damage repair rate defect. ATM-knockdown cells were eventually able to repair the DNA damage fully within 2 h, whereas this was achieved within 1 h in the control cells, highlighting that ATM-deficient cells are able to repair oxidative DNA damage albeit at a slower rate. Since we hypothesized that this defective DNA repair was due to the inability of ATM-deficient cells to elevate cellular PNKP protein levels in response to oxidative stress, we transfected these cells with a mammalian expression plasmid for PNKP. This leads to an elevation in the total protein levels of PNKP in these cells, which was equivalent to that observed in mock-siRNA treated cells following DNA damage induction. Consequently, we demonstrated that expression of PNKP is able to partially reverse the DNA damage repair defect seen in cells in the absence of ATM alone. Specifically, between 10 and 60 min post-treatment with hydrogen peroxide, the ATM-depleted cells complemented with PNKP (light blue bars) show less accumulation of DNA strand breaks and alkali labile sites, visualized by the in-gel DNA repair comet assay, than ATM-depleted cells alone (purple bars). However, expression of PNKP is still unable to fully correct the DNA damage repair defect, since these cells still showed increased levels of DNA strand breaks and alkali labile sites compared to mock-siRNA treated cells (dark blue bars), particularly between

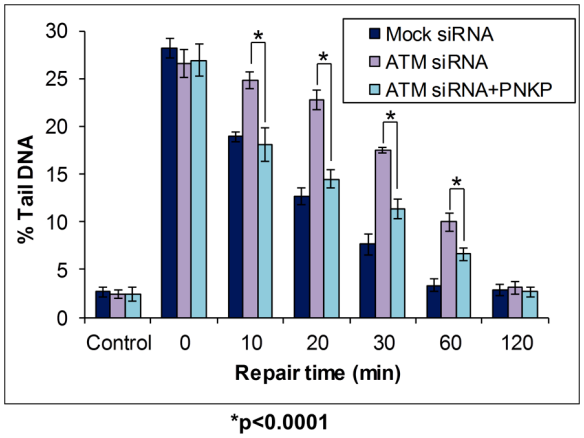

FIGURE 6 | Modulation of BER through Cul4A–DDB1–STRAP-dependent regulation of PNKP protein levels, as revealed by the in-gel DNA repair comet assay. HCT116p53$^{+/+}$ cells were treated in the absence or presence of ATM siRNA for 24 h. Cells were then further treated in the absence (Mock siRNA and ATM siRNA) and presence (ATM siRNA + PNKP) of a mammalian expression plasmid expressing Flag-tagged PNKP for a further 24 h. Cells were analyzed using the in-gel DNA repair comet assay following treatment in suspension with 35 μM hydrogen peroxide for 5 min, and allowing for DNA damage repair. Mean % tail DNA values with SDs were calculated. Further details are provided in **Figure 4** legend. Statistically significant results comparing ATM siRNA versus ATM siRNA-treated cells transfected with a plasmid containing Flag-tagged PNKP are represented by *$p < 0.0001$, as analyzed by Student's *t*-test. Data taken and modified from Parsons et al. (2012).

20 and 60 min post-treatment. Nevertheless, this study demonstrated that ATM-dependant phosphorylation of PNKP, which is required to elevate the levels of PNKP through inhibition of ubiquitylation dependent proteasomal degradation, is required for the efficient repair of DNA damage induced by oxidative stress.

REGULATION OF OTHER BER PROTEIN LEVELS

In addition to Pol β and PNKP, there is accumulating evidence that other BER protein levels are regulated through ubiquitylation-dependent degradation by the UPP, and therefore we will summarize some of the key important findings. APE1, the major AP endonuclease activity employed in BER, has been shown to be polyubiquitylated on lysines 24, 25, and 27 by the mouse double minute 2 (MDM2) E3 ubiquitin ligase (Busso et al., 2009), which is also the major E3 ubiquitin ligase involved in the regulation of the p53 tumor suppressor protein. It was shown by transfection of HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells with an expression plasmid for APE1, that the protein was ubiquitylated in a p53-dependant manner in the presence of DNA damage, and that increased APE1 protein was evident following MDM2 siRNA knockdown. Although more recently, UBR3 was suggested as the major E3 ubiquitin ligase purified from cell extracts that ubiquitylates APE1, within the N-terminus of the protein (Meisenberg et al., 2012). Indeed, *Ubr3*$^{-/-}$ mouse embryonic fibroblasts displayed increased cellular levels of APE1 and were found to be genetically unstable. Both XRCC1 and DNA ligase IIIα, which are known to form a stable complex in human cells that performs the final ligation step in the short patch BER pathway, have independently been shown to be polyubiquitylated *in vitro* by the E3 ubiquitin ligase CHIP (Parsons et al., 2008). This study demonstrated that the levels of both proteins increased following CHIP depletion by siRNA in HeLa cells, demonstrating that CHIP also regulates the stability of these proteins *in vivo*. DNA glycosylases that perform the initial excision step of damaged DNA base removal are also increasingly being identified as targets for ubiquitylation-dependant proteasomal degradation. Specifically OGG1, which is the major enzyme involved in the excision of the mutagenic base 8-oxoguanine, has been shown to be a target for CHIP ubiquitylation, but only in response to hyperthermia due to the protein undergoing thermal unfolding (Fantini et al., 2013). The DNA glycosylase MutYH, that excises adenine residues opposite 8-oxoguanine that are misincorporated during DNA replication of 8-oxoguanine:cytosine base pairs, has been shown to be a target for Mule ubiquitylation both *in vitro* and *in vivo* (Dorn et al., 2014). It was observed that an siRNA knockdown of Mule in HEK293T cells caused an elevation in the cellular levels of MutYH, and that a ubiquitylation deficient mutant of MutYH was similarly more stable following transfection into HEK293T cells. It should also be noted that there is plentiful evidence highlighting an important role for modification of thymine DNA glycosylase (TDG) with the small ubiquitin modifier (SUMO; Hardeland et al., 2002; Steinacher and Schar, 2005; Smet-Nocca et al., 2011). Whilst this topic is beyond the scope of the current review, SUMOylation of TDG has been shown to regulate its DNA glycosylase activity, rather than cellular protein levels. However there is recent evidence suggesting that TDG levels are also regulated by ubiquitylation, although the E3 ubiquitin ligase catalyzing ubiquitylation-dependent degradation of TDG is currently unknown (Moriyama et al., 2014). Finally, the cellular protein levels of DNA polymerase λ which is a close relative of Pol β, has also been shown to be a target for ubiquitylation-dependant degradation initiated by Mule. DNA polymerase λ protein degradation was discovered to be inhibited by Cdk2/cyclin A-dependant phosphorylation in late S and G2 phases of the cell cycle, which promotes recruitment of DNA polymerase λ to chromatin to assist in the repair of 8-oxoguanine DNA base damage (Markkanen et al., 2012).

CONCLUDING REMARKS

In this review, we have highlighted the increasing number of studies demonstrating a vital role for the UPP in the modulation of the cellular steady state levels of key BER proteins. These mechanisms coordinate moderate increases (∼20–50%) in BER protein levels in the cellular response to DNA damage. This mechanism of BER protein regulation is ultimately performed by substrate specific E3 ubiquitin ligases and deubiquitylation enzymes that either add ubiquitin moieties onto target proteins, or conversely remove them, and therefore modulate their degradation by the 26S proteasome. Interestingly, misregulation of BER proteins is frequently observed in several human disorders, such as in aging, cancer, and neurodegenerative diseases. Therefore the next goal will be to examine the role of the UPP, and the enzymes therein, in this disease-dependent misregulation that may reveal the mechanistic processes involved. Particularly in the case of human cancer, this

research may also uncover novel cellular targets for drugs or small molecule inhibitors, which when combined with radiotherapy and/or chemotherapy, may generate novel therapeutic strategies for curing the disease. In this review, we have also described our use of a modified comet assay, where DNA repair activities are monitored by allowing cultured cells to repair in-gel prior to cell lysis and DNA electrophoresis. This method has allowed us to monitor changes in BER regulation via modulation of enzymes involved in the UPP, and has been key to demonstrating the effect of this process in coordinating an efficient cellular response to DNA damage. The modified comet assay has also enabled us to avoid the extensive cell culture-based processing associated with the more traditional approach using attached cells, as discussed earlier. This technique allows for the determination of much more precise DNA repair kinetics at various time points post-treatment (i.e., with hydrogen peroxide or ionizing radiation) and is our preferred method for measuring the repair of DNA strand breaks and alkali-labile sites in the various cell lines that we routinely use.

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Functional evaluation of DNA repair in human biopsies and their relation to other cellular biomarkers

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Thousands of DNA lesions are estimated to occur in each cell every day and almost all are recognized and repaired. DNA repair is an essential system that prevents accumulation of mutations which can lead to serious cellular malfunctions. Phenotypic evaluation of DNA repair activity of individuals is a relatively new approach. Methods to assess base and nucleotide excision repair pathways (BER and NER) in peripheral blood cells based on modified comet assay protocols have been widely applied in human epidemiological studies. These provided some interesting observations of individual DNA repair activity being suppressed among cancer patients. However, extension of these results to cancer target tissues requires a different approach. Here we describe the evaluation of BER and NER activities in extracts from deep-frozen colon biopsies using an upgraded version of the *in vitro* comet-based DNA repair assay in which 12 reactions on one microscope slide can be performed. The aim of this report is to provide a detailed, easy-to-follow protocol together with results of optimization experiments. Additionally, results obtained by functional assays were analyzed in the context of other cellular biomarkers, namely single nucleotide polymorphisms and gene expressions. We have shown that measuring DNA repair activity is not easily replaceable by genomic or transcriptomic approaches, but should be applied with the latter techniques in a complementary manner. The ability to measure DNA repair directly in cancer target tissues might finally answer questions about the tissue-specificity of DNA repair processes and their real involvement in the process of carcinogenesis.

Keywords: *in vitro* comet-based DNA repair assay, base excision repair, nucleotide excision repair, human solid tissue, methodological report

INTRODUCTION

The ability of cells to protect against a large variety of DNA disruptions is a vital process for living organisms. Base excision repair (BER) and nucleotide excision repair (NER) belong to the subgroup of DNA repair mechanisms that are active on structurally modified DNA bases. The biological significance of both pathways is highlighted by the well-known association of BER or NER deficiency with the incidence of inherited (Cleaver et al., 2009) and sporadic types of cancer (Slyskova et al., 2012a). Moreover, the individual's BER and NER capacity is expected to have an influence on the response to anti-neoplastic drug treatment (Pallis and Karamouzis, 2010; Lord and Ashworth, 2012). Therefore, being able to screen an individual's repair capacity may represent a step toward risk assessment and individualized cancer therapy.

Our current knowledge of DNA repair indicates that this process involves many genes that have to work in a synchronized and coordinated way. The simultaneous participation of other processes such as DNA damage signaling, cell cycle controls, and maybe even other (un)known genes, makes DNA repair a multigene and multipathway process. There is a body of evidence concerning different levels of DNA repair gene regulation. The majority of DNA repair genes are polymorphic in the human population, with as yet uncharacterized functional consequences (Ricceri et al., 2012). Therefore, DNA sequence analyses cannot be sufficiently informative for predicting DNA repair activity. Gene expression has been shown to be a misleading source of information, because changes in mRNA levels do not necessarily reflect changes in enzyme activity and vice versa (Damia et al., 1998; Vogel et al., 2000; Paz-Elizur et al., 2007; Stevens et al., 2008; Hanova et al., 2011; Slyskova et al., 2012a,b). This is due to extensive post-transcriptional and post-translational modifications and protein–protein interactions that take part in regulating the activity of repair proteins (Fan and Wilson, 2005; Hu and Gatti, 2011; Nouspikel, 2011). Moreover, DNA repair is a multifactorial process that is modulated not exclusively by genetic background, but, to a certain extent, might be regulated by environmental and lifestyle factors (Wu et al., 2006; Collins et al., 2012). Measuring the true phenotypic endpoint seems in this context to be the most informative, straightforward, and perhaps the most reliable way of characterizing the DNA repair processes.

Both excision pathways follow a common pattern: recognition of the DNA lesion, excision of the damage, and resynthesis of the removed sequence. Transient strand breaks (SBs) are generated as intermediates during both repair pathways, which make BER and NER easily measurable on the functional level by methods based on the comet assay. This assay is a sensitive technique for quantification of SBs in DNA which is applicable not only to measure basal DNA damage, but also in a modified form for measuring DNA repair incision activity. So far, human blood has been used in the majority of studies examining DNA repair activity, since blood is usually the only tissue that can be sampled from healthy subjects. Methods for evaluation of BER and NER from peripheral blood cells are currently well-established (Collins et al., 2001; Langie et al., 2006). However, often it is important to consider the level of DNA repair in particular organs, especially when analysing DNA repair activity in association with tissue-specific diseases. Although it might be logistically complicated in most cases, from cancer patients, there is still a possibility to obtain not only peripheral blood, but also surgically resected normal or tumor tissue. Langie et al. (2010, 2011) have published protocols modified for assessment of DNA repair activity in animal solid tissues; however, a methodological approach for its evaluation in human biopsies has not yet been optimized. Moreover, despite the undeniable biological significance of DNA repair, DNA repair activity is still not routinely included as a biomarker in human biomonitoring studies. This is partially due to the fact that it is a relatively laborious method, especially when large numbers of samples are analyzed.

This text presents a detailed comet assay-based protocol for measuring BER- and NER-specific incision activity *in vitro* from deep-frozen human solid tissues, covering all its optimization steps. The protocol has been recently applied for the first time on colorectal cancer biopsies (Slyskova et al., 2012b). In order to increase the applicability of this approach to large-scale epidemiological studies, the 12-minigel format (12 agarose minigels per microscopic slide) instead of the conventional format (one or two large agarose gels per slide) has been applied. In addition, the relationship between the detected DNA repair activity and other biomarkers (single nucleotide variants in and expression of DNA repair genes) routinely measured in human biomonitoring studies is also discussed.

MATERIALS AND METHODS

STUDY POPULATION AND COLLECTION OF BIOLOGICAL SPECIMEN

The study was conducted on colorectal tissues collected from 70 CRC patients at the surgical resection of the tumor. Patients were recruited between 2009 and 2011 in Thomayer Hospital (Prague), General University Hospital (Prague), and Teaching Hospital and Medical School of Charles University (Pilsen). All patients gave informed consent. Ethics approval was granted by appropriate committees of the three hospitals. The group of patients included 53 men and 17 women with a mean age of 66.2 $\pm$ 10.6 years. The clinical stage of patients at diagnosis was classified according to the tumor-node-metastasis (TNM) system. Seven patients were diagnosed with TNM stage I (10%), 29 as stage II (41.4%), 15 as stage III (21.4%), and 19 as stage IV (27.2%). All patients had adenocarcinomas; 44 patients had tumor localized in the colon (62.9%) and 26 in the rectum (37.1%). In 12 (17.2%) patients, tumors were of well-differentiated grade, in 47 (67.1%) moderately differentiated and in 11 patients (15.7%) poorly differentiated. Eleven rectal cancer patients (15.7%) received neoadjuvant therapy prior to surgery. Tumor tissue and adjacent healthy colon/rectal tissue (5–10 cm distant from the tumor) were resected from all patients. Colon biopsies were briefly washed in PBS and snap frozen immediately after the resection and further stored at −80°C. Prior to tissue processing, histological analysis was carried out to assess the proportion of tumor cells in tumor tissues and to rule out the presence of neoplastic cells in the normal mucosal tissues. The cut-off point was set to 80% of tumor or normal cells in the sample, respectively. Samples were embedded in optimal cutting temperature compound (Sakura Finetek), and cut with a Leica CM 1850 cryostat. Five μm thick serial sections were fixed in 90% ethanol on microscope slides and stained with 1% cresyl violet acetate (Sigma-Aldrich), dehydrated with ethanol, dried, and inspected using a Leica DM6000 microscope (Leica). Due to various logistical reasons, not all patients could be analyzed for all the studied parameters. Therefore, each particular analysis is further specified for actual number of cases for whom analysis was carried out.

IN VITRO COMET-BASED DNA REPAIR ASSAY

Principle of the assay

Protein extracts isolated from human tissues were incubated with substrate DNA in the form of nucleoids, containing artificially induced lesions known to be repaired specifically by either the BER or NER pathway. The photosensitizer Ro 19-8022, in the interaction with visible light, gives rise to oxidative DNA damage (predominantly to 8-oxoguanines; 8-oxoG) that are specifically recognized by the BER machinery. Alternatively, nucleoids containing ultraviolet light (UV)-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts represent the substrate for the NER pathway. The level of induced lesions can be detected by enzymes of bacterial or viral origin, which serve as positive controls in each experiment. Two types of enzymes were used, formamidopyrimidine DNA glycosylase (Fpg) as a prokaryotic analog of human OGG1 that recognizes oxidized purines, and T4 Endonuclease V (Endo V) produced by T4 bacteriophage, recognizing UV-induced CPD. In addition, each experiment included a negative control, namely lesion-containing substrate DNA incubated with reaction buffer to assess the background damage together with buffer-induced damage. Furthermore, each protein extract was measured for (i) specific repair activity (i.e., extract incubated with lesion-containing DNA) and (ii) non-specific endonuclease activity (i.e., specificity control; extract incubated with lesion-free DNA). To be able to record only specific activity of repair proteins, the non-specific endonuclease activity of the protein extract was subtracted. The frequency of DNA SBs, generated during incision of lesions, reflects the DNA repair activity of the extract.

Substrate DNA

In this protocol, the cellular source of substrate DNA consisted of peripheral blood mononuclear cells (PBMC) and human-derived lymphoblastoid cells (TK6), though in principle any

other mammalian cells in suspension could be used. Cells should be controlled for low basal level of SBs (ideally not higher than 10% DNA in tail) and such was the case in this study. PBMC were separated on Histopaque-1077 (Sigma-Aldrich), counted, evaluated by trypan blue exclusion and suspended in ice-cold PBS. TK6 cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 0.2 mg/mL sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich). Cells were counted and suspended in ice-cold PBS. For BER, PBMC were treated with 2μM Ro 19-8022 (Hoffmann-La Roche) for 5 min, and irradiated on ice at 33 cm distance from a 500 W halogen lamp. For NER, TK6 cells were irradiated with 5 Jm^{-2} of UVC (50 s at 0.1 $Jm^{-2}s^{-1}$). Lesion-free PBMC and TK6 cells were prepared in parallel. Cells were aliquoted at 5×10^5 in 0.5 mL of freezing medium (RPMI 1640, 20% fetal bovine serum, 0.2% antibiotics, 10% DMSO, Sigma-Aldrich) and frozen slowly to −80°C. Before each experiment, cells were thawed by adding 1 mL of cold PBS, spun at 400 g, 5 min, 4°C, and suspended in 400 μL of PBS. An 80 μL aliquot of the cell extract was mixed with 260 μL of 1% LMP agarose to reach the desired concentration of cells (∼300–600 cells per minigel). Using a multi-dispensing pipette, twelve 5 μL minigels of cells in agarose were placed on each microscope slide. Cells embedded in agarose underwent lysis for 1 h in 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 250 mM NaOH, 1% Triton X-100, pH 10. Before incubation with protein extracts, slides were washed twice for 5 min with buffer B (45 mM HEPES, 0.25 mM EDTA, 0.3 mg/mL BSA, 2% glycerol, pH 7.8) and placed in incubation chambers (Severn Biotech) (Shaposhnikov et al., 2010).

Protein extracts preparation

Tissue resections were weighed and ground while frozen, and 30–50 mg aliquots were stored at −80°C. For extraction, a volume of 100 μL of buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 7.8) was added to every 50 mg of ground tissue. Samples were vortexed, snap frozen, and 30 μL of 1% Triton X-100 in buffer A added per 100 μL. Protein concentration was measured by a fluorescamine assay (Sigma-Aldrich), on a NanoDrop 3300 (Thermo Scientific). Undiluted extracts were kept at −80°C. Before the incubation reaction, on the day of use, extracts were diluted to a protein concentration of 3 mg/mL in buffer A in a final volume of 50 μL and mixed with 4 volumes of buffer B.

BER-specific reaction

A 30 μL aliquot of extract was added to each minigel in the incubation chamber. Each extract was incubated with Ro-treated as well as non-treated PBMC (used for background subtraction). Incubation time was 20 min, at 37°C in a humid environment. Fpg was used as a positive control. For a negative control substrate DNA was incubated with buffer A + buffer B in a 1:4 ratio. Each experimental point was performed in duplicate. In optimization experiments, PARP inhibitor ABT-888 (Selleckchem) was added to the extract at a concentration of 5 μM to test the effect of inhibiting the post-incision phase of BER.

NER-specific reaction

For the NER-specific assay, the protein extract was enriched with adenosine-5′-triphosphate at a final concentration of 2.5 mM. A 30 μL aliquot of extract was added to each minigel in the incubation chamber. Each extract was in parallel incubated for 30 min with UV-treated and non-treated TK6 cells (used for background subtraction). UV substrate incubated with Endo V was used as positive control and 1:4 buffer A + buffer B as negative control. In optimization experiments, aphidicolin (DNA polymerase delta inhibitor; Sigma-Aldrich) at a concentration of 2.5 μM was added to the extract to test the effect of DNA resynthesis inhibition.

Single cell gel electrophoresis

After the incubation period, the protocol followed was the same as previously described for the comet assay (Olive and Banath, 2006). In brief: slides were treated for 20 min under alkaline conditions (0.3 M NaOH, 1 mM EDTA, pH 12) to allow DNA denaturation and subsequently electrophoresed for 20 min at 1.3 V/cm. Washing followed, with PBS, then H_2O and finally ethanol, each for 10 min. Slides were stained with SYBRGold (Invitrogen) at the concentration recommended by the manufacturer in a bath at 4°C with agitation. After 40 min, SYBRGold solution was removed and the slides rinsed twice with water and left to dry at room temperature. On the day of analysis gels were hydrated by adding a drop of water on top of each minigel and covered with a coverslip. The comets were evaluated by visual scoring performed exclusively by one person (Azqueta et al., 2011). Comets were analyzed by a Nikon fluorescence microscope using 5 classes of comets from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). Hundred comets were selected at random for each sample (50 comets per duplicate gel), so the overall score from one sample ranged from 0 to 400 arbitrary units. Final DNA repair activity was calculated as the difference between scores for treated substrate incubated with extract and non-treated substrate incubated with extract. (Visual scoring was preferred only because of technical problems with image analysis software at the time of the study; however, scoring with computerized software is equally recommended).

GENOTYPING

Single nucleotide polymorphisms (SNPs) were selected according to their (i) location in a gene involved in a pre-incision complex of BER or NER whose activity is detectable by DNA repair assays, (ii) minor allelic frequency >5%, and (iii) predicted damaging or deleterious effect on protein function by SIFT or PolyPhen algorithms (Xi et al., 2004). DNA was isolated from total blood by the phenol-chloroform method. SNPs were detected by TaqMan® SNP Genotyping Assays based on allele-specific TaqMan® MGB probes plus PCR primers and analyzed on Applied Biosystems 96-well real-time PCR instrumentation (Life Technologies). Functional SNPs in BER genes were represented by *OGG1* Ser326Cys (rs 1052133). Selected SNPs within NER genes involved *XPA* G23A (rs 1800975), *XPC* Ala499Val (rs 2228000) and Lys939Gln (rs 2228001), *XPD* Lys751Gln (rs 13181), *XPG* Asn1104His (rs 17655) and *XPF* Arg415Gln (rs 1800067).

REVERSE TRANSCRIPTION qPCR

Tissue samples were homogenized in the MagNA Lyser (Hoffmann-La Roche). AllPrep DNA/RNA mini kit (Qiagen) was used to isolate nucleic acids. Total RNA was measured on ASP-3700 Spectrophotometer (Avans-Biotechnology) for quantity and OD260/280 ratio. RNA integrity number (RIN) was checked using Agilent Bioanalyzer 2100, with RNA 6000 Nano Assay (Agilent Technologies). cDNA was synthesized from 500 ng of RNA using a RevertAidTM First strand cDNA synthesis kit (Thermo Scientific) using random hexamers and following manufacturer's instructions. cDNA was diluted to 10 ng/μL and preamplified for 18 cycles on a CFX96 Real Time PCR Instrument (Biorad) according to the manufacturer's protocol. qPCR was performed using the high-throughput platform BioMark™ HD System (Fluidigm). Ten μL of reaction mix contained 1 μL of 20× diluted preamplified cDNA, 2.5 μL of Taqman Universal Mastermix II without UNG (Life Technologies), 5 μL of primer/probe assays with Perfect Probe^{TM} (Primer Design) at a final concentration of 300 nM, 2.5 μL of 2× Assay loading reagent and 0.25 μL of 20× GE sample loading reagent (Fluidigm) and 1.25 μL of water. Cycling conditions for qPCR were: 95°C for 10 min, 45 cycles of 95°C for 15 s and 50°C for 60 s. *TOP1* and *18S rRNA* were reference genes selected from a geNorm^{TM} reference genes selection kit (Primer Design) by Normfinder algorithm (GenEx Enterprise software). Data were collected from one 48 × 48 array. Data were normalized to reference genes, converted to relative quantities and transformed to log2 scale.

STATISTICAL ANALYSIS

Statistical analysis was performed by SPSS Statistics 18 (IBM) and by GenEx Enterprise (MultiD) softwares. The distribution of investigated parameters was controlled by Kolmogorov–Smirnov test. Expression data were logarithmically transformed to achieve a normal distribution. Two-tailed *T*-test or ANOVA for differences between groups for normally distributed data was employed and correlations determined by a Pearson's test. When data were not distributed accordingly to a Gaussian curve, non-parametric tests of Kruskal–Wallis, Mann–Whitney or Spearman's correlation coefficient were used. All statistical tests were performed at a 95% confidence level.

RESULTS AND DISCUSSION

OPTIMIZATION OF THE BER- AND NER-SPECIFIC ASSAYS

An advanced medium-throughput 12-minigel format

In order to be able to process a larger number of samples and to suppress the effect of inter-experimental variability, we have utilized the 12-minigel format that was introduced by Shaposhnikov et al. (2010) and is demonstrated in **Figure 1**. The comparability of the new 12-minigel approach (12 minigels of 5 μl agarose per slide) with the conventional 2-gel format (2 large gels of 70 μl agarose per slide) was tested by Azqueta et al. (2012). Therefore, we have directly optimized the BER- and NER-repair assays for a 12-minigel format, without any additional testing.

The inter-experimental variability given by the 12-minigel format was low, with the coefficient of variation between 7 independent experiments being 7.7% for both BER and NER (calculated from the negative and positive control, data not shown). This suggests that only up to 8% of variability might be attributed to inter-experimental variations. The 12-minigel format, with its 6-times higher yield of analyzed samples per microscopic slide, significantly increases the applicability of repair assays to human epidemiological studies. Sixteen samples can be optimally run for both assays in one experiment, using only 9 microscopic slides and 9 incubation chambers (see scheme displayed in **Figure 1**). This capacity is not limited by the dimensions of the electrophoretic tank, as is usually the case with the 2-gel format. Another advantage is that the new format requires considerably lower numbers of substrate cells. Instead of ∼3000 substrate cells per gel, the 12-minigel format requires only a tenth of this quantity.

Precision of the assays

To test repeatability of the assays, we have measured 25 samples in two independent experiments and compared the results. As shown in **Figure 2**, the inter-experimental variation is negligible and both assays are repeatable with high reliability ($p \leq 0.001$).

The assays were subsequently tested for the ability to recognize protein extracts deficient in the incision step of repair. For that purpose, extracts from OGG1- and XPG-deficient cells were isolated and their activity compared with the extracts isolated from cells of the same origin but expressing both genes. **Figure 3** presents observed results in comparison to positive and negative controls, as described in detail in *BER- and NER-specific reaction* sections. While protein extracts from wild-type cells showed activity significantly higher than activity measured for knock-out cells (BER: $p = 0.007$, NER: $p = 0.019$), the low activity of knock-out cells was not different from the unspecific activity of buffer only (BER: $p = 0.44$, NER: $p = 0.39$). Both assays confirmed a sensitivity to distinguish biological variability.

Dependence of protein extract activity on protein concentration

We prepared tissue extract dilutions in the range 0 to 18 mg/mL protein content and measured the corresponding activity. Surprisingly, the relation between protein concentration and activity of the extract was not (log-)linear (higher protein amount corresponding with higher activity), but instead exhibited an increase of incision activity reaching its maximum at a protein concentration of 3 mg/mL, followed by a drop of activity with further increasing content of proteins (**Figure 4**). At the concentration point of 3 mg/mL the ratio between lesion-specific activity and non-specific endonuclease activity of the extract was the highest and in favor of the former. Another confounder would be represented by the ratio between protein amount and accessibility of DNA lesions. Too high protein concentration leads to saturation of the reaction. However, protein concentration optimal for the assays was shown to be tissue-specific, as studied on animal tissues (Langie et al., 2010, 2011), and therefore the concentration set by us is not generally applicable. The optimal concentration should be tested by each user of the assay on particular biological samples. Time of incubation is also a variable that can be recommended, but anyway should be pre-tested on each particular substrate DNA with specific extracts.

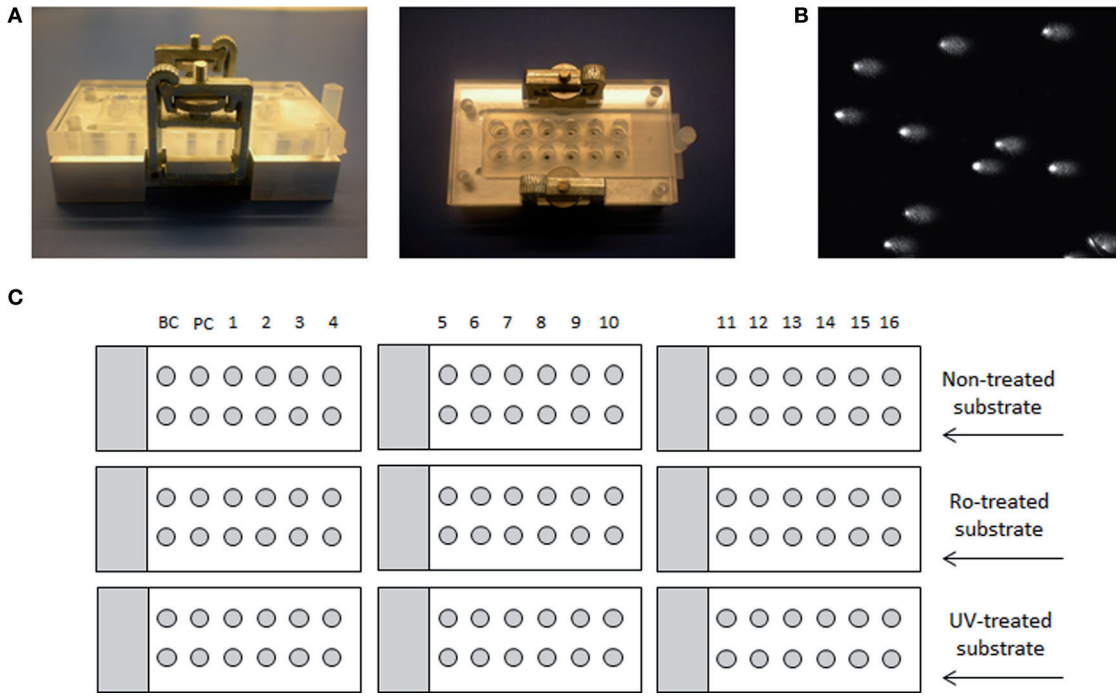

FIGURE 1 | Medium-throughput comet assay format and layout of the experiment. (A) Device for 12-minigel format, **(B)** image of comets, and **(C)** a schematic example of an experiment, using 16 different extracts, background control with buffer only (BC) and a positive control with specific enzyme (PC). The experimental layout with 9 microscope slides is applicable only if the same substrate cell-type is used in each assay, so that the same non-treated cells can be used for both.

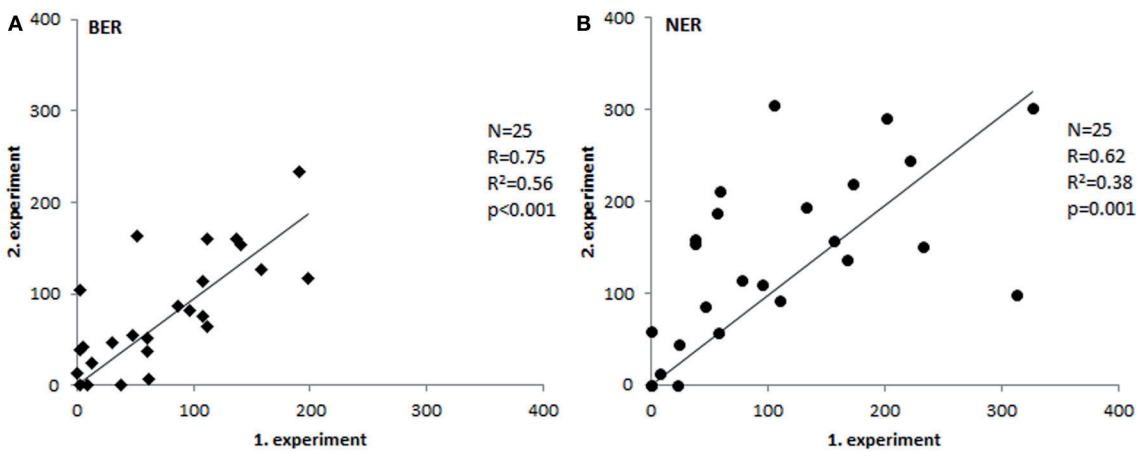

FIGURE 2 | Inter-experimental variability. Comparison of tissue extract activities measured in two separate experiments (Spearman's correlation coefficient). Paired *T*-test *p*-values were 0.58 for BER **(A)** and 0.1 for NER **(B)**.

Do intermediate SBs reflect the incision activity?

To test the postulate that SBs measured in the assays are generated specifically by the incision activity of the protein extract, we have used specific agents to inhibit post-incision phase of the repair processes. ABT-888 is a well-known inhibitor of the Poly-(ADP)-ribose polymerase (PARP) and has therefore been used in the BER assay. The post-incision NER phase was blocked by aphidicolin (APC), inhibiting the function of polymerase delta. On adding the specific inhibitors, all SBs generated by incision activity are expected to remain "open." As **Figure 5** documents, the tissue extracts correspond in 85–88% of their activities irrespective of the presence of the inhibitors. Thus, BER and NER assays detect specifically the incision step of the whole repair process, which is regarded the rate-limiting step (Collins, 1987; Shivji et al., 1992). It involves proteins that are rather active in the repair of DNA damage, unlike post-incision complexes that take part also in the replication or transcription of DNA. The extract is not able to perform the synthetic stages of repair, unless deoxyribonucleotides, and ATP are provided (Collins et al., 1994).

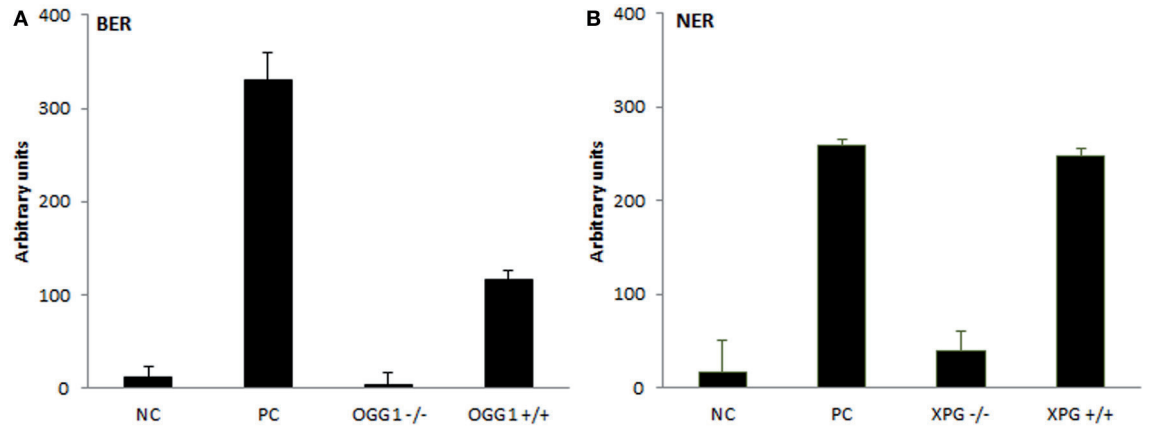

FIGURE 3 | Testing the extracts from DNA repair gene knock-out cells. Measurement of BER and NER activities in knock-out cells and wild-type control cells of the same origin. For **(A)** BER assay extracts were isolated from OGG1$^{-/-}$ and OGG1$^{+/+}$ mice livers; negative control (NC) represents incubation with buffer only and positive control (PC) incubation with Fpg. For **(B)** NER assay extracts were isolated from XPG$^{-/-}$ and XPG$^{+/+}$ hamster ovarian cancer cells CHO AA8, and NC and PC represent incubation with buffer and Endo V, respectively. Data represent means ± *SD* of duplicate measurements.

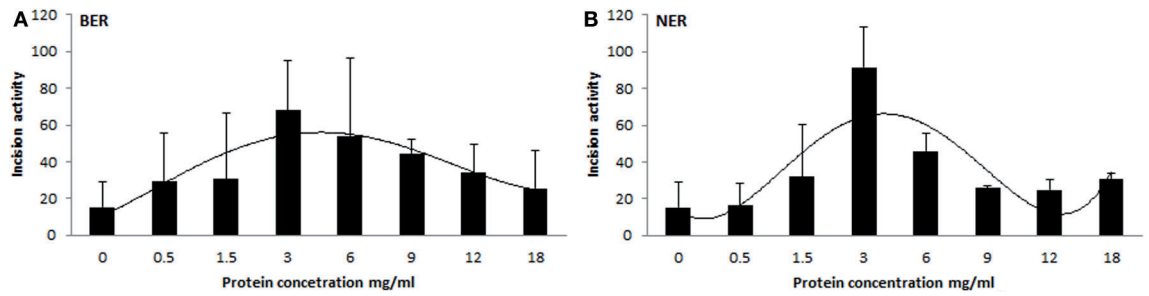

FIGURE 4 | Relationship between protein concentration of extract and its activity. Different protein concentrations of tissue extracts plotted against their BER **(A)** and NER **(B)** activities. Each experimental point represents mean ± *SD* of duplicate measurements.

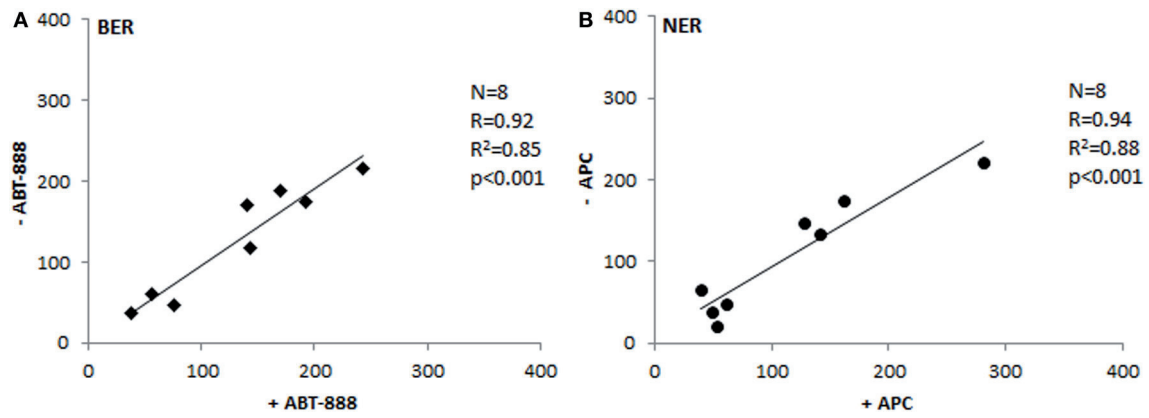

FIGURE 5 | Testing the inhibition of post-incision phase. Comparison of BER **(A)** and NER **(B)** activities between extracts treated with inhibitors of polymerization and the same extracts not suppressed for the polymerization activity (Spearman's correlation coefficient). ABT-888, inhibitor of Poly-(ADP)-ribose polymerase; APC, aphidicolin - inhibitor of DNA polymerase delta.

DNA REPAIR CAPACITY IN RELATION TO OTHER CELLULAR BIOMARKERS

Genotype–phenotype interactions

Ro-induced oxidative damage is mainly represented by 8-oxoG. There are several enzymes known to be specialized for this particular lesion; however the 8-oxoguanine DNA glycosylase (OGG1) is the primary enzyme recognizing and incising this lesion. Among others, NEIL1 and NEIL2 have marginal activity in repair of this lesion, NTH1 repairs free 8-oxoG and MUTYH recognizes adenine already mispaired with 8-oxoG. Therefore, BER

Table 1 | Influence of single nucleotide polymorphisms in BER and NER genes on the BER and NER incision activity (Comparison of medians by Kruskal–Wallis or Mann–Whitney *U*-tests).

SNP	Genotype	*N*	Healthy tissue (*N* = 68)				Tumor tissue (*N* = 68)			
			BER median (quartiles)	*p*-value	NER median (quartiles)	*p*-value	BER median (quartiles)	*p*-value	NER median (quartiles)	*p*-value
OGG1 Ser326Cys (rs1052133)	GG	41	15.3 (9.2–20.8)		12.5 (8.0–23.5)		16.9 (10.8–23.8)		19.1 (14.1–27.9)	
	GC	26	14.8 (8.2–23.5)		14.2 (7.3–18.6)		22.3 (12.8–27.6)		18.3 (7.9–21.2)	
	CC	1	6.2	0.49	15.5	0.79	5.6	0.056	20.4	0.22
	GC + CC	27	14.7 (7.2–23.2)	0.87	14.5 (8.0–18.5)	0.54	22.2 (12.3–27.6)	0.072	18.5 (8.3–21.2)	0.09
XPA G23A (rs1800975)	GG	23	16.1 (10.7–21.6)		14.9 (9.3–20.4)		22.4 (16.7–27.0)		18.7 (16.1–25.0)	
	GA	33	14.5 (8.6–20.2)		11.9 (5.7–23.5)		16.3 (11.2–23.8)		19.1 (8.7–27.2)	
	AA	12	10.4 (3.4–22.5)	0.50	18.9 (11.2–21.3)	0.36	13.9 (1.9–25.3)	0.056	18.9 (10.5–30.0)	0.87
	GA + AA	45	14.1 (7.0–20.7)	0.27	12.5 (7.8–22.4)	0.79	16.3 (10.3–23.8)	**0.018**	19.1 (9.4–27.2)	0.6
XPC Ala499Val (rs2228000)	GG	34	15.3 (8.7–20.5)		14.8 (8.8–24.0)		17.1 (11.2–26.0)		19.2 (11.0–28.9)	
	GA	26	14.7 (9.1–22.0)		14.2 (5.5–18.9)		21.5 (13.8–25.3)		18.4 (11.7–24.5)	
	AA	8	15.6 (6.0–26.3)	0.92	12.2 (6.5–18.8)	0.76	13.6 (1.2–23.0)	0.34	18.9 (9.3–34.4)	0.92
	GA + AA	34	14.7 (8.5–23.4)	0.87	13.7 (5.8–18.9)	0.46	18.7 (12.0–24.7)	0.82	18.4 (10.5–25.7)	0.74
XPC Lys939Gln (rs2228001)	TT	30	14.1 (5.9–21.2)		13.7 (8.2–20.4)		18.7 (9.0–24.7)		18.3 (10.5–24.5)	
	TG	28	15.0 (10.0–22.4)		14.6 (3.3–23.7)		17.1 (12.3–25.4)		18.4 (10.5–31.5)	
	GG	10	16.5 (9.2–22.0)	0.42	13.4 (6.7–17.9)	0.92	19.5 (11.9–27.1)	0.73	19.2 (14.2–23.7)	0.85
	TG + GG	38	15.9 (9.6–21.2)	0.25	14.6 (6.7–21.6)	0.82	17.4 (12.3–25.6)	0.56	18.9 (12.5–28.9)	0.57
XPD Lys751Gln (rs13181)	GG	28	15.6 (9.0–21.5)		14.5 (8.5–24.4)		18.0 (12.6–24.1)		17.1 (9.3–24.8)	
	GT	29	14.5 (7.7–24.0)		13.9 (8.1–20.9)		21.8 (11.8–26.9)		20.8 (14.6–32.1)	
	TT	11	14.9 (12.3–16.3)	0.93	13.5 (5.2–18.9)	0.84	12.5 (5.6–22.2)	0.38	17.5 (13.9–20.4)	0.19
	GT + TT	40	14.7 (8.6–18.3)	0.76	13.7 (7.7–20.3)	0.67	17.2 (11.2–26.5)	0.89	19.6 (14.0–28.1)	0.15
XPF Arg415Gln (rs1800067)	GG	59	15.3 (8.5–23.0)		14.5 (8.3–20.4)		17.7 (11.3–25.8)		18.5 (10.5–26.0)	
	GA	9	14.1 (9.1–18.2)	0.57	13.5 (4.8–26.1)	0.82	18.1 (7.0–23.4)	0.64	22.2 (14.3–26.2)	0.43
	AA	–	–	–	–	–	–	–	–	–
	GA + AA	–	–	–	–	–	–	–	–	–
XPG Asn1104His (rs17655)	GG	41	15.9 (9.3–22.7)		13.5 (4.6–20.2)		19.4 (11.8–26.8)		18.7 (10.8–25.3)	
	GC	22	12.2 (5.8–17.1)		16.4 (10.8–23.7)		14.7 (11.2–24.5)		19.5 (10.5–33.3)	
	CC	5	15.9 (7.2–22.1)	0.24	9.1 (7.3–12.3)	0.17	16.3 (0.6–24.2)	0.23	19.1 (12.9–30.0)	0.71
	GC + CC	27	13.9 (5.8–18.5)	0.13	14.5 (9.1–21.6)	0.44	14.9 (10.7–24.2)	0.09	19.1 (10.5–31.7)	0.42

Associations with p-value < 0.05 *are shown in bold.*

activity, as measured *in vitro* in our assay toward Ro-induced lesions, is mainly reflecting the activity of the BER glycosylase OGG1. In contrast to BER, NER enzymes work in large complexes and the minimal requirement for the incision comprises at least 20 proteins.

The majority of BER and NER genes are polymorphic in the population, and over 50% of them have functionally relevant amino acid changes. By applying SIFT or PolyPhen algorithms, several SNPs are predicted to be possibly damaging, damaging, or deleterious, by means of protein function (Xi et al., 2004) and these *in silico* characterizations are also supported by a range of epidemiological and *in vitro* studies. In this study, functional SNPs in BER genes were represented by the commonly studied *OGG1* Ser326Cys, while NER genes were represented by *XPA* G23A, *XPC* Ala499Val and Lys939Gln, *XPD* Lys751Gln, *XPG* Asn1104His and *XPF* Arg415Gln. All of these potentially functional SNPs were genotyped in the cohort of 68 individuals and their effects on BER and NER activity of colorectal tissues were studied. None of the studied SNPs showed any direct association with DNA repair activity in either healthy or tumor tissues, except for *XPA* 23A allele that was associated with lower BER in tumor tissues only (**Table 1**). An association of *XPA* G23A genotype with BER activity in PBMC was observed by Dusinska et al. (2006), although in a relationship opposite to that found by us. Conflicting findings were obtained from studies with Xpa-deficient mice, where XPA seems not to play an important role in oxidative DNA damage repair (Melis et al., 2013). We are aware of low statistical power and risk of type 2 error due to the low number of individuals carrying variant alleles. Nonetheless, reports on genetic variability in relation to DNA repair activity of target tissue (i.e., tissue other than blood) were missing until now.

Is protein activity related to level of gene transcription?

We have measured the amount of OGG1 transcripts in paired tumor-healthy human colorectal tissues and compared it with the BER-related incision activity, which represents mainly OGG1 activity. The activity of the protein was completely independent of the mRNA quantity, with Pearson's correlation coefficient close to 0 for both tumor and normal tissue (**Figure 6**). Lack of a relationship between mRNA level and activity of the protein is not rare in the literature (Damia et al., 1998; Vogel et al., 2000; Paz-Elizur et al., 2007; Stevens et al., 2008; Slyskova et al., 2012a,b). On the contrary, there is growing evidence on the important role of regulation of enzyme activity at post-transcriptional and post-translational levels. *OGG1* is a housekeeping gene of constitutive expression independent of the cell cycle (Dhenaut et al., 2000). It might be regulated via two CpG islands located in the promoter region; however, this was not the case in our samples since none of 88 samples exhibited C methylation in *OGG1* promoter (Slyskova et al., 2012b). OGG1 has eight alternative isoforms/splicing variants of two major groups; type 1 acts in the nucleus, and type 2 in the mitochondria (Boiteux and Radicella, 2000). However, this would not serve as

Table 2 | Correlation of expression of 17 genes involved in NER pre-incision complex with overall NER incision activity (Pearson's correlation coefficient).

Gene	NER activity			
	Healthy tissue ($N = 44$)		Tumor ($N = 44$)	
	R	*p*-value	*R*	*p*-value
CCNH	−0.146	0.35	−0.028	0.86
CDK7	−0.012	0.94	0.320	**0.036**
CSB	−0.101	0.51	−0.039	0.80
DDB1	−0.158	0.31	−0.094	0.54
DDB2	−0.200	0.19	−0.183	0.23
ERCC1	−0.133	0.39	−0.146	0.35
LIG1	0.086	0.58	0.123	0.43
MNAT1	−0.125	0.42	−0.094	0.54
RAD23B	−0.030	0.85	0.190	0.22
RPA1	−0.079	0.61	−0.05	0.75
RPA2	0.007	0.96	0.067	0.67
RPA3	−0.039	0.80	0.225	0.14
XPA	−0.168	0.28	−0.246	0.11
XPB	−0.055	0.73	−0.009	0.96
XPC	−0.062	0.69	−0.004	0.98
XPD	−0.164	0.29	−0.001	0.99
XPF	−0.136	0.38	−0.091	0.56

Associations with p-value < 0.05 *are shown in bold.*

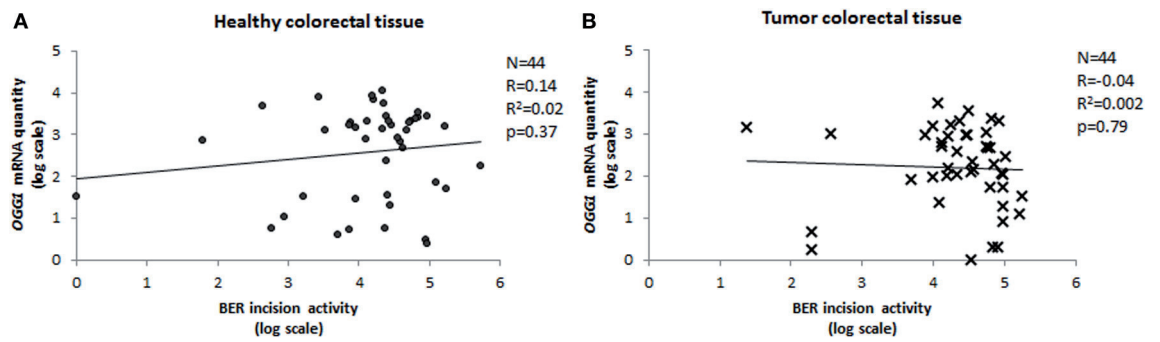

FIGURE 6 | Correlation between *OGG1* expression and BER incision activity. Relative quantity of *OGG1* transcripts measured in 44 paired healthy **(A)** and tumor **(B)** tissue samples plotted against BER activity (Pearson's correlation coefficient).

an explanation of missing mRNA quantity/protein activity correlation either, since all transcript variants have been covered in the assay. Nevertheless, other mechanisms might regulate gene activity; for example 160 microRNAs identified up to now are able to bind to OGG1 transcripts (http://bioinformatics.ekmd.huji.ac.il/reptar/gene_report.php?species=human&id=12458). Above all, two post-translational modifications—phosphorylation and nitrosylation—modulate the final protein activity. Another source of variability might be represented by protein–protein interactions (Fan and Wilson, 2005).

The mRNA expression of the majority of proteins forming the pre-incision complex of NER was also measured and plotted against the overall NER activity. The expression level of none of the 17 studied genes was significantly associated with the NER incision activity, except for *CDK7* protein involved in TFIIH complex in the tumor tissue only (**Table 2**). DNA damage recognition and incision is much more complex in NER as compared to BER. In BER, usually only 1 or 2 proteins are able to recognize and incise damage from DNA, while in NER, the whole complex of many proteins is required for lesion removal. NER proteins work in an interactive downstream manner and are known to be substantially regulated at a post-translational level, which makes the lack of correlation of single gene expression and endpoint NER incision activity understandable. According to our results and the results of other research groups (Damia et al., 1998; Vogel et al., 2000; Paz-Elizur et al., 2007; Stevens et al., 2008; Hanova et al., 2011; Slyskova et al., 2012b), expression analysis of single genes is not a sufficiently informative marker of activity of protein or protein complexes.

CONCLUDING REMARKS

Analyzing DNA repair activity in target tissue might represent an important step toward individualized anti-cancer therapy. Previously we have shown that activities of the BER and NER pathways positively correlate between white blood cells and healthy colon tissue, but not between blood cells and tumor (Slyskova et al., 2012b). Therefore, methods for assessing functionality of DNA repair in solid tissues are warranted. New comet-based repair assays are reliable, simple, fast, and of low cost. An advanced medium-throughput format is suitable for large epidemiological studies. We have also shown that measuring DNA repair activity is not easily replaceable by a genomic or transcriptomic approach, but should be applied with the latter techniques in a complementary manner.

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Epithelial cells as alternative human biomatrices for comet assay

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The comet assay is a valuable experimental tool aimed at mapping DNA damage in human cells *in vivo* for environmental and occupational monitoring, as well as for therapeutic purposes, such as storage prior to transplant, during tissue engineering, and in experimental *ex vivo* assays. Furthermore, due to its great versatility, the comet assay allows to explore the use of alternative cell types to assess DNA damage, such as epithelial cells. Epithelial cells, as specialized components of many organs, have the potential to serve as biomatrices that can be used to evaluate genotoxicity and may also serve as early effect biomarkers. Furthermore, 80% of solid cancers are of epithelial origin, which points to the importance of studying DNA damage in these tissues. Indeed, studies including comet assay in epithelial cells have either clear clinical applications (lens and corneal epithelial cells) or examine genotoxicity within human biomonitoring and *in vitro* studies. We here review improvements in determining DNA damage using the comet assay by employing lens, corneal, tear duct, buccal, and nasal epithelial cells. For some of these tissues invasive sampling procedures are needed. Desquamated epithelial cells must be obtained and dissociated prior to examination using the comet assay, and such procedures may induce varying amounts of DNA damage. Buccal epithelial cells require lysis enriched with proteinase K to obtain free nucleosomes. Over a 30 year period, the comet assay in epithelial cells has been little employed, however its use indicates that it could be an extraordinary tool not only for risk assessment, but also for diagnosis, prognosis of treatments and diseases.

Keywords: comet assay, human epithelial cells, comet sampling

INTRODUCTION

In this special issue, we review the use of the comet assay to map DNA damage in different human cells since the inception of this field nearly 30 years ago by Ostling and Johanson (1984). The aim of the present review is to summarize data published in the meantime that address the use of this tool in evaluating DNA damage in cells other than blood mononuclear cells. An increasing number of studies are being published in this area, particularly with respect to life style, environmental, and occupational exposure risk evaluations, as well as therapeutic interventions, promoting the use of the comet assay as an additional suitable human biomarker.

At the International Workshop on Genotoxicity Test Procedures (IWGTP), which was held in Washington, DC in 1999, an expert panel met to develop guidelines for the use of the comet assay in genetic toxicology. The expert panel reached a consensus that the optimal version of the assay for identifying genotoxic activity was the alkaline (pH > 13) version of the assay that was developed by Singh et al. (1988). This version of the comet assay is capable of detecting DNA single-strand breaks (SSB), alkali labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites. The advantages of the comet assay relative to other genotoxicity tests include its sensitivity in detecting low levels of DNA damage; the requirement of a small number of cells per sample; and its flexibility, ease of application, and short duration. The expert panel identified the minimal experimental and methodological standards required to ensure that the results of comet studies would be accepted as valid by knowledgeable scientists and regulatory agencies (Tice et al., 2000).

It is important to note that only one study addressing human monitoring was published between 1988 and 1993, which was a review article authored by McKelvey-Martin et al. (1993). Subsequently, periodical publications regarding lifestyle and human exposure studies have greatly increased, the majority of which were included in the review articles published in 1999 (Rojas et al., 1999) and 2009 (Valverde and Rojas, 2009).

More recently, the launch of the ComNet project during the International Comet Assay Workshop (ICAW) meeting in Kusadasi, Turkey proposed the aim of establishing the comet assay as a reliable and trusted biomarker assay (Collins et al., 2012). The first ComNet project publication focused on the use of the comet assay as a tool for human monitoring, assuming some difficulty in validating previously published data. The most important disadvantages of the studies were the small number of subjects and the discrepancies in the methodological aspects applied in different

laboratories around the world using blood cells (Collins et al., 2014).

To avoid the previously identified variations resulting from the use of blood cells, the few human biomonitoring comet assay studies using epithelial cells allow us to review the protocols and observe the methodological conditions that optimized their use and enhanced their application.

The present review aims to construct a set of widely acceptable guidelines that may help to eliminate much of the experimental variation that has generated the large heterogeneity of comet assay data and frustrates attempts to compare and combine studies in different laboratories using the comet assay in different types of epithelial cells (specifically, lens, corneal, tear duct, buccal, and nasal epithelial cells).

Epithelial cells, as specialized components of many organs, have the potential to serve as biomatrices that can be used to evaluate genotoxicity and may also serve as early effect biomarkers; furthermore, 80% of solid cancers are of epithelial origin. Epithelial cells are characterized by common structural features (specifically, their arrangement into cohesive sheets), but have diverse functions that are made possible by many specialized adaptations. Many of the physical properties of epithelial cells are dependent upon their attachment to one another, which is mediated by several types of cell junctions. The specialized functions of epithelial cells are mediated through both structural modifications of their surfaces and internal modifications, which adapt cells to fulfill their specific roles, ranging from absorption to secretion to serving as a barrier.

The surface epithelia and the epithelia of many simple glands belong to the continuously renewing cell population. The rate of cell turnover is characteristic of the specific epithelium; for example, small intestinal cells are renewed every 4–6 days in humans. The stratified squamous epithelium of the skin is replaced approximately once every 28 days (Ross and Pawlina, 2006), nasal epithelial cells are replaced approximately once every 30 days and buccal epithelial cells are renewed approximately once every 10–14 days. However, other epithelial cells, particularly those in more complex glands or tissues, may survive for a long time (Kruze, 1994; Ross and Pawlina, 2006; Chiego, 2014).

All of these specialized modifications of the epithelia necessitate various modifications to the comet assay procedure to obtain a single cell suspension, a limiting step in performing the assay using these types of cells.

According to International Program of Chemical Safety (IPCS) guidelines (Albertini et al., 2000), the optimal sample collection timing for any cell population is during long-term chronic exposure when the induction and repair of DNA damage is presumed to be maintained at steady-state equilibrium; such timing maximizes the likelihood that an agent can be identified as DNA damaging. For the sampling of cells after an acute exposure or after termination of chronic exposure to a genotoxic agent, the optimal collection time for detecting induced DNA damage is most likely within a few hours of exposure termination; this window of sampling can affirm that the extent of DNA damage in a population of cells decreases as the amount of time between exposure termination and sampling increases. In addition, the repair of DNA damage through DNA repair processes and the loss of heavily damaged cells through apoptosis, necrosis, or cell turn over are also dependent upon the agent of exposure. An additional advantage of the comet assay for human biomonitoring is the feasibility of its application to a broad spectrum of cells, including both proliferating and non-proliferating cells, as well as cells in tissues that are the first sites in which the genotoxic insult occurs. With the application of the comet assay to these various cell types, a better estimation of risk exposure can be made.

As previously mentioned, the most important details to consider with respect to a single cell suspension that is adequate for analysis using the comet assay include: The sampling protocol, sample storage, sample preparation, and adaptations of the comet assay. These aspects will be discussed in the present review.

THE COMET ASSAY IN LENS EPITHELIAL CELLS

The majority of studies using the comet assay in lens epithelial cells have been conducted in animals (Mitchell et al., 1998; Singh et al., 2002; Bannik et al., 2013; Liu et al., 2013; Aly and Ali, 2014), lymphocytes (Wolf et al., 2008; Liu et al., 2013) or human lens epithelial cell cultures (Lixia et al., 2006; Yao et al., 2008; Pierscionek et al., 2010, 2012; Gao et al., 2011; Liu et al., 2013) (**Table 1**).

When the lens epithelial cells of cataract patients are used directly (Sorte et al., 2011; Øsnes-Ringen et al., 2013; Zhang et al., 2014), the cells must be obtained and dissociated prior to their use in the comet assay.

SAMPLING PROTOCOL AND SAMPLE STORAGE

In the study conducted by Sorte et al. (2011), lens epithelial cells from healthy controls were used after removal of the cornea and anterior capsule using a forceps. Continuous curvilinear capsulorhexis was performed through a clear corneal incision under local anesthesia in senile cataract patients. The anterior capsule was removed via viscoexpression through a clear corneal incision, and the anterior capsule was collected using forceps to avoid direct damage. After removal of the anterior capsule, the samples were maintained in minimum essential media. A single rhexis was placed in Eagle's Minimal Essential Medium containing 10% fetal bovine serum. Zhang et al. (2014) performed the same procedure as Sorte et al. (2011), with some modifications. A continuous curvilinear capsulorhexis was performed through a clear corneal incision under anesthesia. The anterior capsules were immediately removed and placed in Dulbecco's Modified Eagle's Medium containing 15% fetal bovine serum. The maximum amount of time that elapsed between sample collection and the initiation of processing was 30 min in both studies. Øsnes-Ringen et al. (2013) analyzed consecutive capsulotomy specimens obtained from age-related cataract patients. A clear corneal incision was made, viscoelastic material was introduced and the anterior capsule was extracted. The tissue samples were immediately placed in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) containing 15% fetal bovine serum. The samples were analyzed either immediately or after 1 week incubation in the same medium at 37°C in the presence of 5% CO_2.

Table 1 | Variations in comet assay protocols employing human lens epithelial cells.

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Lens	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/8 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min /20 min, 25 V	0.4 M Tris, pH 7.5	Ethidium bromide	Fluorescence microscope and CometScoreTM software	20 comets	% of DNA in the head and in the tail	Sorte et al., 2011
Lens	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/Overnight	30 min	1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/30 min, 1.4 V/cm, 300 mA	PBS	SYBR Gold	Comet Assay IV; Perceptive Instruments	50 comets	% of DNA in the tail	Øsnes-Ringen et al., 2013
Lens	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/Overnight		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 20 V/200 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Fluorescence microscopy and CAPS software	50 comets	% Tail DNA and Olive tail moment	Zhang et al., 2014

COMET ASSAY SAMPLE PREPARATION

Sorte et al. (2011) prepared a cell suspension using mechanical shaking of the capsule (in 50 μl of PBS) by hand for 10–15 min at 4°C to shed lens epithelial cells from the lens capsule, after which point the capsule was discarded. Øsnes-Ringen et al. (2013) and Zhang et al. (2014) prepared suspensions of single cells after pipetting the lens epithelium several times. After the capsule was discarded, the cell suspensions were centrifuged at 200 × g for 5 min at 4°C, the supernatants were discarded and the cells were resuspended in PBS.

COMET ASSAY

In the study conducted by Sorte et al. (2011), the comet assay was conducted according to the procedure developed by Singh et al. (1988), with a few modifications. Cells in the second agarose layer were embedded by mixing equal volumes of the cell suspension (50 μl) with 2% Low Melting Point Agarose (LMPA) instead of 80 μl of 1% LMPA and 20 μl of cell suspension. Zhang et al. (2014) embedded 50 μl of the cell suspension mixed with 100 μl of 0.75% LMPA onto slides that had been pre-coated with 0.75% Normal Melting Point Agarose (NMPA). Øsnes-Ringen et al. (2013) used 30 μl of the cell suspension mixed with 140 μl of 1% LMPA and 10 5 μl drops were placed onto a glass slide (that had been pre-coated with agarose and dried) as two rows of five (in the absence of coverslips) (**Table 1**).

Enzyme treatment

Of the three studies, only Øsnes-Ringen et al. (2013) used lesion-specific enzymes to detect specific types of DNA damage. After lysis, the slides were rinsed three times for 5 min each in enzyme buffer at 4°C. Using a silicone gasket and a plastic chamber (Shaposhnikov et al., 2010), each gel on the slide was isolated and incubated with 30 μl of buffer or enzyme (formamidopyrimidine DNA glycosylase, endonuclease III and T4 endonuclease V). Two gels were incubated with each of the solutions for 30 min at 37°C in a moist chamber (**Table 1**).

RESULTS

Sorte et al. (2011) detected prominent DNA migration in the majority of the cataractous lens epithelial cells, but not in the majority of the control subjects. DNA fragments in the tail of the comets displayed smearing, indicating that chemical damage had occurred.

Øsnes-Ringen et al. (2013) detected low levels of strand breaks, with mean values of DNA in the tails of 0.2 and 0.6% before and after cultivation, respectively.

Zhang et al. (2014) detected comets in the majority of the lens epithelial cells and lymphocytes of age-related cataract patients, as well as in some of the lymphocytes from the control patients, but comets were not detected in the majority of the lens epithelial cells that were derived from control patients. The researchers observed that DNA damage in lymphocytes was more severe than that in the corresponding lens epithelial cells from the same individuals, speculating that systemic, and local oxidative damage might affect each other.

DISCUSSION

DNA damage that was assessed using the comet assay in lens epithelial cells was mainly studied in the context of cataracts. This multifactorial pathogenesis is the major cause of blindness worldwide. Epidemiological, clinical and experimental studies indicate that UV radiation and oxidative stress are significant contributors to the development of lens opacities. In particular, DNA damage and cell death has been demonstrated in lens epithelial cells obtained from patients with cataracts. The low levels of strand breaks that were detected by Øsnes-Ringen et al. (2013) in the age related cataractous lens epithelium may be explained by patient selection and/or by the protocol that was used to obtain and process the samples. Similar considerations at respect to patient selection to those that apply to the variation in results reported from other groups may also apply to this study. The previously discussed investigations call for further studies of DNA damage in human lens epithelial cells from lenses with and without cataracts using the comet assay. In particular, such investigations may provide novel information regarding the mode of DNA damage progression *in vivo*, allow for informed *ex vivo* interventions to reduce damage and/or stimulate damage repair, and ultimately lead to clinical studies of prophylactic approaches.

THE COMET ASSAY IN CORNEAL CELLS

In corneal cells, studies using the comet assay have been conducted in animals (Rogers et al., 2004; Choy et al., 2005; Roh et al., 2008; Morkunas et al., 2011; Jester et al., 2012), lymphocytes of patients (Czarny et al., 2013) or human lens epithelial cell cultures (Wu et al., 2011; Ye et al., 2011, 2012).

When using the cornea directly (Haug et al., 2013; Lorenzo et al., 2013), the cells must be obtained and dissociated prior to use in the comet assay.

SAMPLING PROTOCOL AND SAMPLE STORAGE

In the study conducted by Haug et al. (2013), the corneas were stored in Optisol GS at 4°C prior to transplantation and the remaining corneo scleral rims were acquired for the study. For the comet assay, 10 rims were used. Half of each rim was immediately processed for analysis, while the other half was transferred to Eye Bank Organ Culture (OC) for 1 week prior to analysis. This experimental design was selected to examine the effects of OC on tissue that had been previously stored in Optisol GS. Lorenzo et al. (2013) used human corneo-scleral tissue that was obtained from rings after penetrating keratoplasty and preserved in OC prior to use. The corneo-limbal rings were transferred to dishes containing DMEM/F12, in which the peripheral sclera and cornea were trimmed off. The rings were divided into 12 samples measuring approximately 2 × 2 mm. The samples were washed in Hanks Balanced Salt Solution in the absence of Ca^{2+} and Mg^{2+} at room temperature.

COMET ASSAY SAMPLE PREPARATION

To obtain a single-cell suspension, Haug et al. (2013) removed the epithelium by scraping on ice before gentle pipetting and centrifuging at 200 × g for 5 min at 4°C. The cells were resuspended in PBS. Lorenzo et al. (2013) generated duplicate samples from each ring that were incubated at 37°C in a humid atmosphere containing 5% CO_2 in pre-equilibrated 0.05% trypsin in HBSS containing 0.02% EDTA-$_4$Na (in the absence of Ca^{2+} and Mg^{2+}) for 1 or 3 h in either 250 µl or 3 ml of the solution using 96- or 6-well plates, respectively. At the end of the incubation period, enzyme activity was terminated by adding an equal amount of serum-containing growth medium (DMEM/F12). The cells were dispersed by gentle pipetting. The dissociated cells from each well in media/enzyme solution were transferred to tubes on ice.

COMET ASSAY/ENZYME TREATMENT

Haug et al. (2013) and Lorenzo et al. (2013) performed the comet assay according to the procedure developed by Azqueta et al. (2009), with some modifications. Haug et al. (2013) used lesion-specific enzymes to detect specific types of DNA damage. After lysis, the slides were rinsed in enzyme buffer at 4°C. Using a silicone gasket and a plastic chamber (Shaposhnikov et al., 2010), each gel in the slide was isolated and incubated with 30 µl of buffer or enzyme (formamidopyrimidine DNA glycosylase, endonuclease III, and T4 endonuclease V). The gels were incubated with each of the solutions for 30 min at 37°C in a moist chamber. Untreated lymphocytes were used as a negative control, and lymphocytes from healthy volunteers that had been treated on ice with 2 µM photosensitizer Ro 19-8022 plus visible light (a 500 W tungsten-halogen source at 33 cm) to induce 8-oxoGua were used as a positive control. The control cells were treated in the same manner as corneal epithelial cells, but were incubated with only enzyme buffer or FPG (**Table 2**).

RESULTS

Haug et al. (2013) found that the levels of strand breaks were low in cold-stored tissues. Enzyme-sensitive sites were generally not increased by much in OC, with the exception of certain samples that displayed substantial increases in Endo III-sensitive sites (oxidized pyrimidines); marked increased were observed in 3 of the 10 samples, while the levels of FPG-sensitive sites were similar in the two groups. The levels of T4 endo V sites increased.

In the study conducted by Lorenzo et al. (2013) using trypsin-EDTA, DNA damage was observed in the form of strand breaks, regardless of the volume of enzyme solution and the duration of incubation. A trend toward increased damage was observed when using 3 ml compared to 250 µl. Increasing the incubation time from 1 to 3 h did not consistently increase the levels of strand breaks.

DISCUSSION

Previously, studies of human cells using the comet assay have generally focused on blood cells or cultivated cells.

Little information is available regarding the amounts of molecular damage inflicted upon essential molecular constituents, including DNA, by the different protocols. We report that the comet assay may yield valuable information regarding the amounts and types of DNA damage in such tissues. However, in contrast to blood cells, the cells in these tissues must be dissociated prior to analysis using the comet assay, and such methods may induce various types and amounts of DNA damage.

For the *ex-vivo* storage, culture and engineering of tissues for transplant purposes, one main challenge is to provide tissues in

Table 2 | Variations in comet assay protocols employing human corneal cells.

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Corneal	–/Overnight		–	20 min/20 min, 20 V aprox. 300 mA	PBS 10 min H_2O 10 min	SYBR Gold	Comet Assay IV; Perceptive Instruments	50 comets	% of DNA in the tail	Haug et al., 2013
Corneal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 1.3 V/cm	PBS 10 min H_2O 10 min	SYBR Gold	Visually classified using fluorescence microscopy	100 comets	Overall score ranging from 0 to 400 units	Lorenzo et al., 2013

which the individual cells harbor minimal amounts of molecular damage, in particular DNA damage. The study conducted by Haug et al. (2013) using the corneal epithelium demonstrated that cells may be dissociated from the cornea using mechanical procedures. Subsequent investigations using the comet assay provided information regarding the levels and types of DNA damage under different storage and incubation conditions. Lorenzo et al. (2013) demonstrated that dissociation of corneo-limbal epithelial cells using trypsin-EDTA, a procedure that is commonly used to initiate cultures for the *ex-vivo* engineering of transplantable tissues, is associated with DNA damage.

The protocols used for tissue storage and culture and the *ex-vivo* engineering of tissues for transplant purposes differ between clinics, and various types of nutrient solutions and incubation conditions are commonly used. In addition, cells may be dissociated from the original tissue using enzyme solutions prior to tissue engineering and seeded on a substrate for propagation. Certain protocols call for the positioning of tissue samples directly on the substrate. On such substrates, novel tissue is generated by cells that migrate from the tissue of origin out onto the substrate.

The studies outlined above demonstrate that the comet assay may provide crucial information regarding the integrity of the DNA in such tissues. Such information is of significant value in research aimed at improving *ex-vivo* conditions and the quality of tissues destined for transplantation.

THE COMET ASSAY IN EXFOLIATED TEAR DUCT CELLS

The search for relevant target cells for human monitoring has revealed the potential use of exfoliated tear duct epithelial cells in the comet assay. To date, only one study has applied these cells during comet assay monitoring (Rojas et al., 2000). The main lachrymal gland serves to keep the eye surface clean, using tears to clear the eye of desquamated cells, particles, and diluting gasses or liquids. The gland is located beneath the conjunctiva on the upper lateral margin of the orbit and drains into the upper fornix of the conjunctiva via a series of approximately 10 small ducts. The cells that desquamate into the tear film are those from the cornea, which is covered by stratified, squamous, non-keratinized epithelium with a basal cell layer that gives rise to five to six superficial layers.

SAMPLING PROTOCOL AND SAMPLE STORAGE

A total of 20 μl of tears were obtained using a 20 μl capillary tube from the inner nasal angle of the right eye while nasal brushing was performed, stimulating the olfactory bulb. The samples were maintained in the capillary tubes at room temperature prior to performing the comet procedure.

COMET ASSAY SAMPLE PREPARATION

Epithelial cells contained in a tear film, which served as a physiological solution, did not require special preparation. The samples contained in the capillary tubes were pushed using a gum bulb into a microtube to be mixed with LMPA (0.5%) (**Table 3**).

COMET ASSAY

The alkaline procedure was conducted by pipetting 75 μl of the cell mixtures (tears and LMPA) onto a slide that had been precoated with 180 μl of normal agarose and immediately covered

Table 3 | Overview of the comet assay protocol employing tear duct epithelial cells.

Biomatrix	Lysis solution/duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	Reference
Tear duct	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, pH 10, 1% Triton X-100, 10% DMSO/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 0.8 V/cm	0.4 M Tris pH 7.5	Ethidium bromide	Scaled ocular	50 comets	Tail length Microns	Rojas et al., 2000

with a coverglass to form a microgel, allowing the agarose to jellify. A third LMP (0.5%) agarose layer was added. The slides were immersed in lysis solution (pH 10) for 1 h. The DNA was allowed to unwind for 20 min in electrophoresis buffer, and electrophoresis was then conducted at 0.8 V/cm for 20 min (**Table 3**).

RESULTS

The authors of the study only analyzed 25 nucleoids per slide in duplicate, suggesting a low quantity of cells in 20 μl of tear film. DNA damage increased in the tear duct epithelial cells of individuals exposed to urban atmospheres with high ozone concentrations. Because this is the only study that was conducted using this type of cell, comparison of the procedures is not possible.

DISCUSSION

The study presented data regarding the use of exfoliated tear duct epithelial cells for monitoring. This cell type presents various advantages for monitoring, as follows: The cells can be acquired using minimally invasive procedures; sufficient cells are present in only one tear drop; and these cells are relevant to genotoxicity studies involving cosmetic products, airborne carcinogens, and all agents that may come in contact with the eyes (Rojas et al., 2000).

THE COMET ASSAY IN BUCCAL CELLS

Evaluation of DNA damage in buccal epithelial cells may provide a biomarker of early damage in target tissues. This type of cells has been employed principally in human studies through non-invasive methods and is easily applied as a biomarker in biomonitoring studies in a similar manner to micronuclei.

These cells must be directly obtained from the oral cavity and dissociated prior to use in the comet assay.

SAMPLING PROTOCOL AND SAMPLE STORAGE

In the studies conducted by Rojas et al. (1996), Valverde et al. (1997), Eren et al. (2002), and Beričević et al. (2012), buccal cells were obtained after the use of mouth wash by scraping the internal part of the cheek with a wood or plastic stick and were added to RPMI-1640 medium during transportation prior to being rapidly processed. Faccioni et al. (2003) collected buccal mucosal cells by gently brushing the internal portion of the cheeks using an interdental brush after washing out the mouth many times with tepid water. The brushes were stirred in 5 ml of PBS (pH 7.4). Similarly Szeto et al. (2005), Jayakumar et al. (Jayakumar and Sasikala, 2008), and Mondal et al. (2011) used soft bristle toothbrushes to collect buccal cells by scraping the inside of the cheeks after rinsing the mouth with distilled water. The toothbrushes were then agitated in 30 ml of cold PBS. Ursini et al. (2006) and Cavallo et al. (2006, 2009) also collected exfoliated buccal cells after the subjects had rinsed their mouths with water by scraping the interior of the cheeks with a toothbrush. They suspended the cells in 25 ml of Titenko-Holland buffer containing 0.01 M Tris-HCl, 0.1 M EDTA and 0.02 M NaCl (pH 7), and immediately sent the cells to the laboratory to perform the assay. Westphalen et al. (2008) collected the cells by gentle brushing of the inside portion of the lower lip with a cytobrush after washing the mouth out several times with tepid distilled water. The brushes were stirred in 20 ml of PBS. Sudha et al. (2011)

and Eshkoor et al. (2011, 2013) obtained the cells by gently rubbing the inside of both cheeks with an extra soft toothbrush for 1 min. The brushes were then rinsed in a tube containing 30 ml of saline before finally being washed with PBS (pH 7.4). Pal et al. (2012) obtained the cells using oral brushing after the subjects had washed their mouths with normal saline (a 0.9% NaCl solution). The collected samples were maintained in PBS. Visalli et al. (2013) obtained the oral mucosal cells by scraping the cheeks with a moist wooden spatula. Prior to scraping, the subjects rinsed their mouths with saline.

COMET ASSAY SAMPLE PREPARATION

Various studies generated suspensions of cells that were immersed in RPMI-1640 via centrifugation over a range of 1–10 min at 800–6000 rpm (Rojas et al., 1996; Jayakumar and Sasikala, 2008; Westphalen et al., 2008; Mondal et al., 2011; Sudha et al., 2011). Similarly, Szeto et al. obtained pellets and resuspended them in 100 μl of PBS. Additional procedures were also reported (Faccioni et al., 2003), in which the cell suspensions were centrifuged, suspended in PBS, and filtered through polyamide gauze (with a 100 μm mesh opening). The filtrates were pelleted using centrifugation and resuspended in RPMI-1640. Beričević et al. (2012) centrifuged the cell suspension for 3 min at 3200 rpm and resuspend it in PBS (pH 7.4), after which point cell viability was determined and one aliquot was immediately resuspended in a chilled buffer at pH 7.5 (containing 0.075 M NaCl and 0.024 M Na_2 EDTA). The cells were macerated on ice for 2 min. Visalli et al. (2013) reported that after 1 h, the exfoliated cells were processed at 800 × g for 3 min and the pellets were suspended in 40 μl of PBS. The number of epithelial cells, on average, ranged from 1 to 2×10^6/ml, which equated to 40,000–80,000 cells per subject. However, reports in which exfoliated buccal cells were washed twice in PBS and then suspended in approximately 100 μl of the same buffer in the absence of centrifugation have also been made (Cavallo et al., 2006, 2009; Ursini et al., 2006). In addition, it is important to note that certain groups did not provide data regarding sample preparation because the use of specific kits or details of this manner were not included in the publications(Valverde et al., 1997; Eren et al., 2002; Eshkoor et al., 2011, 2013; Pal et al., 2012).

COMET ASSAY/ENZYME TREATMENT

Only four reports used the original three agarose layers containing the same percentages that were reported by Singh et al. (1988) (i.e., 0.5%) (Rojas et al., 1996; Valverde et al., 1997; Ursini et al., 2006). The majority of reports employed only two agarose layers containing volumes ranging from 70 to 100 μl of LMPA and NMPA, and the percentage of agarose used ranged from 0.7 to 1%.

The majority of the studies that were conducted to determine DNA damage in buccal epithelial cells used the alkaline comet assay according to the procedure developed by Singh et al. (1988), with various modifications. Only the studies that employed the modifications outlined by Szeto et al. (Szeto et al., 2005) performed neutral comet assays (pH 9.1) (Jayakumar and Sasikala, 2008; Mondal et al., 2011; Pal et al., 2012) (**Table 4**).

However, the principal challenge in using this cell type is the cellular modifications that occur in the epithelium; it is thus important to take the enzymatic procedure that is employed during the lysis process to obtain free DNA that can respond to the electrophoretic field in to account. Various studies only used the lysis protocol that was originally proposed by Singh et al. (1988) (0.1 M EDTA, 2.5 M NaCl, 0.01 M Tris, and 1% N-laurylsarcosine, pH 10, with the fresh addition of 1% Triton X-100 and 10% DMSO) (Faccioni et al., 2003; Cavallo et al., 2006, 2009; Ursini et al., 2006; Westphalen et al., 2008; Sudha et al., 2011; Beričević et al., 2012). Certain studies reported the use of lysis conditions that corresponded to those of a specific kit (Eshkoor et al., 2011; Sudha et al., 2011), while other studies utilized a combination of different lysis conditions. However, all of the studies employed proteinase K (broad-spectrum serine protease) digestion under optimal conditions during lysis (Rojas et al., 1996; Valverde et al., 1997; Eren et al., 2002; Szeto et al., 2005; Jayakumar and Sasikala, 2008; Mondal et al., 2011; Pal et al., 2012; Visalli et al., 2013) using the procedure that was first outlined by Szeto et al. (Faccioni et al., 2003), in which proteinase K lysis is achieved using trypsin/EDTA digestion (Jayakumar and Sasikala, 2008; Mondal et al., 2011; Pal et al., 2012) (**Table 4**).

In the reviewed studies using buccal epithelial cells, only the Cavallo et al. (2006) study used FPG to detect oxidative DNA lesions (**Table 4**).

RESULTS

The study conducted by Rojas et al. (1996) employed enzymatic lysis enrichment to compare DNA damage between buccal epithelial cells that were derived from smokers and non-smokers; in spite of the fact that the study employed a small number of subjects; the comet assay was found to be suitable for use in this cell type. In the study conducted by Valverde et al. (1997), DNA damage induced by air pollution in Mexico City was compared in three different cell types, demonstrating that lysed buccal epithelial cells with proteinase K enrichment are suitable for use in comet analyses; however, differences between the exposure groups were not detected. The same protocol was recently employed by Visalli et al. (2013) to determine that subjects with restorative dental fillings (both amalgams and resin-based fillings) displayed genotoxic damage in the oral mucosa. The study conducted by Eren et al. (2002) examined the effects of chlorhexidine in blood and buccal epithelial cells and found that the comet assay in combination with lysis enrichment was able to identify damaged cells with greater sensitivity than the determinations of damage that were conducted in blood cells that had been obtained from the same subjects. In contrast, the study conducted by Westphalen et al. (2008), in which the comet assay was performed in buccal cells in the absence of modifications, did not detect orthodontic appliance-induced DNA damage after 10 days. However, Faccioni et al. (2003) and Beričević et al. (Eren et al., 2002) determined that nickel and cobalt released from fixed orthodontic appliances can induce DNA damage in oral mucosal cells in the absence of modifications to the Singh et al. procedure (Singh et al., 1988). In a similar manner, the studies conducted by Ursine et al. (Mondal et al., 2011) and Cavallo et al. (2009), in which changes to the protocol were not made, obtained negative comet assay results in

Table 4 | Variations in comet assay protocols employing human buccal cells.

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Buccal	2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/48 h	2 h Proteinase K 140 μl (10 mg/ml) 37°C 2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO	1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	100 comets	Tail length Microns	Rojas et al., 1996
Buccal	2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/24 h	1 h Proteinase K 100 μl (10 mg/ml) 37°C 2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO	1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	100 comets	Tail length Microns	Valverde et al., 1997
Buccal	2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO 1 h	2 h Proteinase K 140 mg/ml 37°C	1 mM Na_2EDTA, 300 mM NaOH, pH 13	40 min/40 min	0.4 M Tris, pH 7.5	Ethidium bromide	Visually classified using fluorescence microscopy	100 comets	Cell percentage in three categories	Eren et al., 2002
Buccal	-/Overnight		-	25 min/20 min, 25 V/300 mA (0.86 V/cm)		Ethidium bromide	Comet Assay II; Perceptive instruments	100 comets	Tail length, % Tail DNA, and Olive tail moment	Faccioni et al., 2003
Buccal	30 min 50 μl (0.25% Trypsin, 1 mM EDTA in Hanks balanced salt solution)	1 h Proteinase K (1 mg/ml). 1 h conventional lysis (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO)	1 mM NaOH, 1 mM EDTA, pH 9.1	20 min/18 min, 12 V constant	0.4 M Tris, pH 7.5	Ethidium bromide	Komet 3.0 software	Two gels per donor	Tail DNA %	Szeto et al., 2005

(Continued)

Table 4 | Continued

Biomatrix	Lysis solution/ Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Buccal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 V/300 mA, 20 min	0.4 M Tris, pH 7.5	Ethidium bromide	Image analyzer Delta Sistemi	50 comets	Tail moment	Ursini et al., 2006
Buccal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/1 h	30 min FPG (1 μg/ml)	1 mM Na_2EDTA, 300 mM NaOH, pH 13	40 min/30 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Image analyzer Delta Sistemi	50 comets	Tail moment	Cavallo et al., 2006
Buccal	-/Overnight	-	-	25 min/20 min, 25 V/300 mA (0.86 V/cm)	-	Silver nitrate	Light microscope	50 comets	Overall score of between 0 and 400 arbitrary units	Westphalen et al., 2008
Buccal	30 min 50 μl (0.25%Trypsin, 1 mM EDTA in Hanks balanced salt solution)	1 h Proteinase K (1 mg/ml). 1 h conventional lysis (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO)	0.01 M NaOH 1 mM EDTA, pH 9.1	20 min/18 min, 12 V(constant)	0.4 M Tris, pH 7.5	Ethidium bromide	Komet 5.5 software		Tail DNA % Tail length Olive tail moments	Jayakumar and Sasikala, 2008
Buccal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 V/300 mA, 20 min/20 min	0.4 M Tris, pH 7.5	Ethidium bromide	Image analyzer Delta Sistemi	50 comets	Tail length Tail DNA % Tail moment	Cavallo et al., 2009
Buccal	-/1 h	-	-	-	-	Ethidium bromide	Image analysis s system	100 comets	Tail length microns	Sudha et al., 2011

(Continued)

Table 4 | Continued

Biomatrix	Lysis solution/ Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Buccal	30 min 50 μl (0.25%Trypsin, 1 mM EDTA in Hanks balanced salt solution)	1 h Proteinase K (1 mg/ml). 1 h conventional lysis (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO)	0.01 M NaOH, 1 mM EDTA, pH 9.1	20 min/18 min, 12 V constant	0.4 M Tris, pH 7.5	Ethidium bromide	Komet 3.0 software	Two gels per donor	Tail DNA %	Mondal et al., 2011
Buccal	Trevigen lysis/1 h	-	-	45 min/10 min, 1 V/cm^{-1}	-	SYBR Green	Tri Tek comet score 1.5	-	Tail length	Eshkoor et al., 2011
Buccal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/72 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	10 min/16 min, 300 mA (0.66 V/cm)	0.4 M Tris, pH 7.5	Ethidium bromide	Comet assay IV software	100 comets	Tail DNA % Tail length	Beričević et al., 2012
Buccal	30 min 50 μl (0.25%Trypsin, 1 mM EDTA inn Hanks balanced salt solution)	1 h Proteinase K (1 mg/ml).	10 mM NaOH, 1 mM EDTA, pH 9.1	10 min/20 min, 30 V constant	0.4 M Tris, pH 7.4	Ethidium bromide	Komet 5.5 software	100 comets	Tail DNA % Olive tail moment	Pal et al., 2012
Buccal	Trevigen lysis/1 h	-	-	45 min/10 min, 1 V/cm^{-1}	-	SYBR Green	Tri Tek comet score 1.5	-	Tail length	Eshkoor et al., 2013
Buccal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/ Overnight	Proteinase K 8 μl (10 mg/ml) 15 min, 40 °C	1 mM Na_2EDTA 300 mM NaOH pH 13	20 min/40 min, 300 mA 25 V (0.86 V/cm)		Ethidium bromide	Image analysis system CASP	50 comets	Tail DNA %	Visalli et al., 2013

buccal epithelial cells in healthcare workers handling antineoplastic drugs. In contrast, other studies performed in the absence of lysis modification obtained increased DNA migration using the comet assay in buccal epithelial cells to determine the effects of exposure to polycyclic aromatic hydrocarbons (PAHs) (Cavallo et al., 2006, 2009) among metal welders (Sudha et al., 2011) and mechanical workshop employees (Eshkoor et al., 2011, 2013). The study was performed according to the major procedural changes that were outlined by Szeto et al. (2005), and the modifications enabled the application of the comet assay in human biomonitoring and nutritional studies. This group proposed that successful lysis can be achieved using 0.25% trypsin for 30 min followed by proteinase K (1 mg/ml) treatment for 1 h and electrophoresis at a neutral pH (0.01 M NaOH and 0.001 M EDTA, pH 9.1). They induced H_2O_2-mediated DNA damage in a dose-dependent manner and observed Trolox (a water–soluble analog of vitamin E) protection. They also demonstrated that *in situ* exposure to antioxidant-rich green tea diminished DNA strand breaks. The same procedure was applied in buccal epithelial cells by Jayakumar and Sasikala (2008), who observed increased levels of DNA damage among jewelry workers and demonstrated the synergistic ability of cigarette smoking to induce DNA damage. Mondal et al. (2011) also employed the modifications outlined by Szeto et al. (2005) to demonstrate the induction of buccal epithelial DNA damage in women chronically exposed to biomass smoke. The report by Pal et al. (2012), which employed the same procedure, demonstrated that tobacco-associated DNA damage in the oral mucosa was decreased following the regular consumption of black tea.

DISCUSSION

Buccal epithelial cells may be considered to be short-lived cells (with renewal of 10–14 days) due to their continued renewal, while in comparison, peripheral blood lymphocytes may be considered to be longer-lived cells. Therefore, the presence of buccal cells with comet-like appearances is indicative of recent exposure to various substances. This consideration may explain the higher levels of DNA damage that were observed in buccal epithelial cells after exposure to agents that came into direct contact with the oral mucosa, the molecular mechanisms underlying which are closely related to the oxidative DNA damage that is induced by air pollutants and the inflammation that is triggered by the use of orthodontic apparatus.

The use of buccal epithelial cells to determine genotoxicity using the comet assay according to the procedure outlined by Singh et al. (1988) was limited by the inability to obtain free nucleoids. Originally, the enrichment of lysis solution with proteinase K was proposed to eliminate cellular- and nuclear-associated proteins to obtain nucleoids that would migrate in the electric field during alkaline electrophoresis (Rojas et al., 1996; Valverde et al., 1997; Eren et al., 2002). The previously mentioned studies employed RMPI-1640 as a vehicle to maintain buccal epithelial cells, and used similar concentrations of proteinase K during lysis. However, after these studies, confidence in the procedure that was used to conduct the comet assay with buccal epithelial cells began to decrease due to inconsistencies in the sampling procedure, which justified the use of protocols that did not employ lysis modifications in contrast to studies that incorporated many modifications to the procedure (Rojas et al., 1996; Valverde et al., 1997; Eren et al., 2002; Faccioni et al., 2003; Szeto et al., 2005; Cavallo et al., 2006; Ursini et al., 2006; Jayakumar and Sasikala, 2008; Westphalen et al., 2008; Cavallo et al., 2009; Eshkoor et al., 2011; Mondal et al., 2011; Sudha et al., 2011; Beričević et al., 2012; Pal et al., 2012; Eshkoor et al., 2013). However, it is important to note that the studies conducted by Szeto et al. (2005), Jayakumar and Sasikala (2008), Mondal et al. (2011), and Pal et al. (2012) revealed different types of DNA fragments due to the use of neutral conditions followed by the unwinding and electrophoresis procedures. Thus, these results are only comparable to those of studies that conducted the same procedure (**Table 4**).

Future reports should include images that support the use of the comet assay in buccal epithelial cells, as well as details regarding sampling and the manner in which the cell suspension was handled prior to lysis. The use of a non-invasive method to obtain buccal epithelial cells and the potential to determine genotoxicity in cells that come in to direct contact with the potential insult are important aspects that are required to validate the use of a procedure during the alkaline comet assay.

THE COMET ASSAY IN NASAL CELLS

The search for relevant target cells that can be used to study the genotoxic effects of xenobiotics has increased over the past few years. In this context, nasal tissue cells are the first to come in contact with environmental xenobiotics. Exfoliated mucosal cells have been postulated to have predictive value for the detection of carcinogens because 90% of human tumors are of epithelial origin (Cairns, 1975). The comet assay has been examined as a suitable and rapid screening method to determine chemical substance-induced DNA damage in human nasal mucosal cells (Pipkorn et al., 1988).

SAMPLING PROTOCOL AND SAMPLE STORAGE

The studies performed using this cell type typically apply one of three different sampling protocols. The first sampling protocol is the classical method of obtaining cells from the lower edge of both lower nasal turbinates using a disposable nylon brush or cytobrush under direct visual inspection; this method is neither painful nor invasive (Pipkorn et al., 1988; Calderon-Garcidueñas et al., 1996). The nasal epithelium that was obtained using this procedure was immediately immersed in 1 ml of cold RPMI-1640 medium. All of the samples were collected at the same time and rapidly processed. This procedure was used by Calderon-Garcidueñas et al. (Valverde et al., 1997; Calderon-Garcidueñas et al., 1999; Glück and Gebbers, 2000; Kleinsasser et al., 2001; Tisch et al., 2002, 2005; Fortoul et al., 2003a,b, 2004, 2010; Gosepath et al., 2003; Pacini et al., 2003; Koreck et al., 2007; Hölzer et al., 2008; Ginzkey et al., 2012).

Another sampling protocol obtained the cells via nasal epithelial biopsies (Kleinsasser et al., 2001; Gosepath et al., 2003; Tisch et al., 2005; Hölzer et al., 2008; Ginzkey et al., 2012). Following blood clot removal and the proteolytic separation (50 mg of protease, 10 mg of hyaluronidase, and 10 mg of collagenase) of mucosal cells, Tisch et al. (Gosepath et al., 2003) adjusted the cell

number to 1×10^6 cells/ml in Joklik medium. In a study conducted by Gosepath et al. (Sassen et al., 2005), after harvesting and mincing the biopsy specimens, the specimens were trypsinated in pronase for 24 h at 4°C and digested for 15 min at 37°C. The cells were then washed in a phosphate buffer solution and centrifuged. In a study conducted by Tisch et al. (Hölzer et al., 2008), the tissue was incubated with a proteolytic enzyme solution in a shaking water bath at 37°C for 60 min. Fetal calf serum (FCS) was added to avoid uncontrolled enzyme activity. Meanwhile, Hölzer et al. (Reiter et al., 2009) performed mucosal cell disintegration via enzymatic digestion (50 mg of protease, 10 mg of hyaluronidase, and 10 mg of collagenase in 10 ml Ham's F12) for 30 min. Digestion was terminated by centrifugation (10 min at 276 × g), removal of the enzyme solution, and resuspension of the cells in culture medium.

Recently, Ginzkey et al. (Baumeister et al., 2009a) performed another protocol, in which nasal mucosal specimens were obtained during human nasal passage surgery. The nasal mucosa was separated from the bone and connective tissue via enzymatic digestion in a manner that differed from that used in other studies [100 µl of enzyme mix containing 0.1 g of protease and 1.0 mg of DNase dissolved in 10 ml of phosphate buffered saline were prepared using 9 ml of Airway Epithelial Growth Medium (AEGM)]. The specimens were incubated with enzymes for 24 h on a shaker at 4 °C. After terminating the enzymatic reaction with FCS, the cell suspension was filtered through sterile gauze, and washed twice with PBS. Cell number and viability were assessed using the trypan blue exclusion test.

A third method involved the sophisticated generation of 3D miniorgan cultures of human inferior turbinate epithelia (MOCs) from nasal biopsies (Baumeister et al., 2009b; Hackenberg et al., 2010, 2011; Koehler et al., 2010, 2013). Following immediate transport to the laboratory, the cells were minced in 25 × 5 mm pieces and washed three times in Bronchial Epithelial Growth Medium (BEGM) and placed in 24-well plates(one fragment per well). The wells were coated with 0.75% agar noble that had been dissolved in Dulbecco's Modified Eagle Medium containing 10% FCS and non-essential amino acids, streptomycin, and amphotericin B. MOCs floated in 250 µl of BEGM per dish at 37°C, 5% CO_2, and 100% relative humidity. Adhesion to the dish surface was prevented using agarose. BEGM was renewed every other day, and the multiwell plates were replaced on days 7 and 9 to renew the agarose. After 5 days, the initial mucosal fragments were completely coated with partly ciliated epithelium (Baumeister et al., 2009a,b; Hackenberg et al., 2011; Koehler et al., 2010, 2013). In Sassen et al. (2005) and Hackenberg et al. (2010), a similar protocol was performed, with the difference being the use of Airway Epithelial Cell Growth Medium (AECGM) in place of BEGM. In addition, Buehrlen et al. (Hackenberg et al., 2011) used Bronchial Epithelial Basal Medium (BEBM) in place of BEGM. Both authors used penicillin in place of amphotericin B.

Recent studies conducted by Koehler et al. (2010, 2013) and Hackenberg et al. (2010, 2011) utilized a new biopsy handling protocol. Upon receipt, the specimens were cleaned of blood and cartilage by washing in Minimum Essential Medium to isolate the epithelial cells from the specimens, and the cells were then incubated for 24 h in a mixture of 10 ml MEM that had been supplemented with 0.1 mg/ml of protease XIV, 1 mg/ml of DNAse DN25 and antibiotics (0.05 mg/ml of gentamicin, 100 U/ml of penicillin containing 1 µg/ml of streptomycin, 0.250 U/ml of amphotericin B and 2 ml of glutamine). After 24 h, the enzyme activity was terminated by adding 5 ml of FCS. The cells were then scratched from the specimen with a scalpel and poured into a dish. This cell suspension was centrifuged at 500 × g for a duration of 5 min. The cell pellets were resuspended in 1 ml of AECGM that had been supplemented with antibiotics (100 U/ml of penicillin and 1 µg/ml of streptomycin). Cell viability was assessed by vital staining with 0.4% trypan blue, and the number of cells was determined using a light microscope. The human nasal cells were cultured on porous membrane inserts (0.4 µm Corning® Transwell polycarbonate membrane inserts; 12 mm diameter). The porous membrane inserts were covered with 150 µl of collagen I (66 ng/ml), incubated for 3 h at 37°C in a humidified incubator and then stored at 4°C until use. A total of 10^4 epithelial cells were cultured in the BEGM suspension and pipetted onto single membrane inserts. Additional media was added until a minimum of 1.5 ml of BEGM was apical to the membrane and 2 ml was present beneath the membrane in the well. The plates containing the membranes were cultured at 37°C in a humidified incubator in the presence of 5% CO_2. The cells attached to the membrane within 2–3 h. The media was changed every 48 h and the membranes were washed with 2 ml of PBS during the media exchange. After reaching 70–80% confluence on day 7, the media that was apical to the membrane was removed and nutrition was provided to the cells by adding 1.3 ml of BEGM per insert under the membrane. At this point, the cultures achieved air-liquid interface conditions, which were maintained from days 7 to 14 to stabilize the culture conditions. Media exchange beneath the membrane and apical rinsing of the membranes with 2 ml of PBS were carried out three times per week.

COMET ASSAY SAMPLE PREPARATION

The nasal epithelium that was obtained using the cytobrush was immediately immersed in 1 ml of cold RPMI-1640 medium. The nasal samples were easily dispersed into single cells by gently shaking the glass tubes. The single nasal cell suspension volume was then adjusted to 50,000 cells/50 µl of medium (Calderon-Garcidueñas et al., 1996, 1999; Rojas et al., 1996; Fortoul et al., 2003a,b, 2004, 2010). Concurrently, Pacini et al. (Fortoul et al., 2003a) soaked and shook the nylon brush in 2 ml of ice-cold, oxygenated (5% CO_2) minimum essential medium that had been supplemented with 10% FCS. The released cells were maintained on ice and in the dark for no longer than 2 h and were subsequently centrifuged at 250 × g at 4°C for 10 min. The resulting pellets were resuspended in 100 µl of ice-cold medium, and the nasal cell suspension volume was adjusted to prepare the comet slides.

In contrast, the nasal cells that were obtained from biopsies were treated after this exposure period; the viability of the cultures was examined using trypan blue and the cultures were then centrifuged for 10 min at 400 rpm. Once the obtained cell pellets had been resuspended in 1 ml of fresh medium, the final cell suspension was available (Kleinsasser et al., 2001; Tisch et al.,

2005; Hölzer et al., 2008). It is also important to mention that some of the groups did not provide information regarding sample preparation (Gosepath et al., 2003; Ginzkey et al., 2012).

Following xenobiotic treatment, the MOCs were enzymatically digested by incubation for 45 min at 37°C with collagenase P (1 mg/ml), hyaluronidase that had been isolated from bovine testes (1 mg/ml) and pronase E (5 mg/ml) that had been dissolved in BEGM. The enzymes were neutralized using FCS, and the cells were washed twice in cold PBS.

COMET ASSAY/ENZYME TREATMENT

Comet slide preparation varied with respect to agarose layer number, agarose percentage, and volume. In spite of these differences, similarities in the nasal cell procedures were also identified; for instance, nearly all of the studies that obtained samples using a cytobrush applied three agarose layers (0.5% NMPA was used for the first layer, followed by 0.5% LMPA for the second and third layers) (Valverde et al., 1997; Fortoul et al., 2003b, 2004; Koreck et al., 2007). Pacini et al. (Fortoul et al., 2003a) employed three agarose layers, all of which consisted of LMPA; however, information regarding the concentrations used was not reported. Gluck and Gebbers (Pacini et al., 2003) also omitted these details. Calderon-Garcidueñas (Calderon-Garcidueñas et al., 1999; Glück and Gebbers, 2000) reported the use of two agarose layers (0.5% NMPA followed by LMPA), without specifying the concentrations used. Studies in which the nasal epithelial cells were obtained from biopsies typically utilized three layers, with the exception of Gosepath (Tisch et al., 2005), which only reported the use of one LMPA layer, and Ginzkey (Kleinsasser et al., 2001), which reported the use of two layers(1.5% NMPA followed by 0.5% LMPA). In the studies in which three layers were used, the first layer consisted of 1% NMPA, while the second and third layers consisted of 0.7% LMPA (Ginzkey et al., 2012). In the studies conducted by Tisch et al. (Gosepath et al., 2003; Hölzer et al., 2008), the first layer consisted of 1% NMPA, the second and third layers consisted of 0.5% LMPA, and the cells were embedded in the third layer. All of the studies that were performed using MOCs utilized only two agarose layers; in nearly all of these studies, the first layer consisted of 0.5% NMPA and the second layer consisted of 0.7% LMPA (Kleinsasser et al., 2004; Sassen et al., 2005; Buehrlen et al., 2007; Baumeister et al., 2009a,b; Reiter et al., 2009; Koehler et al., 2010); differences in the agarose concentration and the composition of the first (1.5% NMPA) and second (0.5% LMPA) layers were applied by Koehler et al. (Hackenberg et al., 2010) 85]. The studies conducted by Hackenberg (Hackenberg et al., 2011; Koehler et al., 2013) only mention that the cells were embedded in LMPA (**Table 5**).

The remainder of the comet assays that were performed in nasal cells all followed the alkaline version of the protocol that was proposed by Singh et al. (1988), with very few modifications. After generating the slides, the cells were exposed to a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2 EDTA, and 10 mM Tris-base, pH 10, containing 10% DMSO, and 1% Triton X-100) for a minimum of 1 h; however, certain studies did not include the addition of sodium sarcosinate, DMSO or Triton X-100. Enzyme addition to detect specific DNA damage was only performed in two of the studies; Koreck et al. (Fortoul et al., 2010) used the enzyme UVDE to detect cyclobutane pyrimidine dimers, and Baumeister et al. (Koehler et al., 2010) used formamidopyrimidine glycosylase, which specifically recognizes 8-hydroxy-guanines (**Table 5**).

To allow the DNA to unwind, an alkaline electrophoresis buffer was used; during this step, 70% of the studies used 1 mM Na_2 EDTA and 300 mM NaOH (pH > 13), while the remaining 30% employed a buffer containing 200 mM Na_2 EDTA and 5–10 mM NaOH (Kleinsasser et al., 2001; Buehrlen et al., 2007; Baumeister et al., 2009a; Hackenberg et al., 2010, 2011; Koehler et al., 2013). With respect to the duration of unwinding and electrophoresis, 90% of the studies utilized 20 min for unwinding and 20 min for electrophoresis, applying a current of 25 V and 300 mA (ranging from 0.8 to 1 V/cm). Following electrophoresis, the alkaline conditions were neutralized using a 0.4 M Tris (pH 7.5) solution. The slides were subsequently stained with ethidium bromide, with the exception of one study, in which SYBR Green was used (Gosepath et al., 2003) (**Table 5**).

Evaluation of the slides was performed using either scaled ocular or specialized software to measure tail length (45%), % tail DNA (11%) and Olive tail moment (41%), with the exception of one study, which measured only the tail length and width (3%) (Tisch et al., 2005). The number of comets evaluated per slide ranged from 50 to 153 (**Table 5**).

RESULTS

As mentioned previously, comet assay studies utilizing human nasal epithelial cells may be divided into three groups based on the sampling procedure that was used: Cytobrush-obtained, biopsies and MOCs. Methodologically, the nasal cell studies using direct sampling from subjects approached the comet assay in similar manners (Valverde et al., 1997; Calderon-Garcidueñas et al., 1999; Glück and Gebbers, 2000; Tisch et al., 2002; Fortoul et al., 2003a,b, 2004, 2010; Pacini et al., 2003; Koreck et al., 2007). The majority of the studies that were used to determine air pollution-induced DNA damage obtained positive results using the comet assay; specifically, certain studies also revealed a correlation between DNA damage and ozone exposure (Valverde et al., 1997; Calderon-Garcidueñas et al., 1999; Glück and Gebbers, 2000; Tisch et al., 2002; Fortoul et al., 2003a; Koreck et al., 2007). Two of the studies that were conducted by Fortoul et al. (2003b, 2004) detected increased DNA damage in nasal epithelial cells from asthmatic individuals. Meanwhile, the study conducted by Koreck et al. (Fortoul et al., 2010) determined that more DNA damage was induced by phototherapy and utilized a repair assay to determine that the induced damage was removed after 10 days; it is also important to mention that this study was the only study to utilize enzymatic digestion to analyze specific DNA lesions.

Five studies utilized nasal cavity biopsies, and all of these studies established primary cultures that were treated *ex-vivo*. Gosepath et al. (Tisch et al., 2005) did not report the use of enzymatic digestion of the specimens to obtain the cell suspensions that were used in the comet assay. This study reported the induction of DNA damage following benzene treatment for a period of 8 h, and this damage persisted after 24 h. Tisch et al. (Gosepath et al., 2003; Hölzer et al., 2008) examined the genotoxicity of

Table 5 | Variations in comet assay protocols employing human nasal epithelial cells.

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V	0.4 M Tris, pH 7.5	Ethidium bromide		100 comets	Tail length Damage categories	Calderon-Garcidueñas et al., 1996
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/24 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	100 comets	Tail length (microns)	Valverde et al., 1997
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100/1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V 300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	100 comets	Tail length Damage categories	Calderon-Garcidueñas et al., 1999
Nasal	pH 10	-	pH 13	-	-	Ethidium bromide	Scaled ocular	25 representative microscope fields	Tail length	Glück and Gebbers, 2000
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/1 h	-	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 0.8 V/cm	0.4 M Tris, pH 7.5	Ethidium bromide	Custom-made imaging software	100 comets	% Tail DNA	Pacini et al., 2003
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Na sarcosinate pH 10 1 h		1 mM Na_2EDTA, 30 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA, 0.8 V/cm	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	50 comets	Tail length	Fortoul et al., 2003a
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10 1 h		1 mM Na_2EDTA, 30 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA, 0.8 V/cm	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	50 comets	Tail length	Fortoul et al., 2003b
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10 1 h		1 mM Na_2EDTA, 30 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA, 0.8 V/cm	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	50 comets	Tail length	Fortoul et al., 2004
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100/1 h	UVDE enzyme 1.30 h	1 mM EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris - HCl, pH 7.4	Ethidium bromide	Komet 5.0 Kinetic Imaging	100 comets	% Tail DNA	Koreck et al., 2007

(Continued)

Table 5 | Continued

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
Nasal	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10 1 h		1 mM Na_2EDTA, 300 mM NaOH, pH 13	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Scaled ocular	100 comets	Tail length	Fortoul et al., 2010
Nasal epithelial biopsies	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100/1–24 h	50 mg protease, 10 mg hyaluronidase, 10 mg collagenase in Juklick medium	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Kinetic Imaging	153 per condition/3 slides	Tail length	Tisch et al., 2002
Nasal epithelial biopsies	-/45 min	Trypsinated in pronase	_	45 min/10 min, 1 V/cm, 300 mA	-	SYBR green	Adobe photoshop	100 comets	Images Tail length Tail width	Gosepath et al., 2003
Nasal epithelium biopsies Nasal epithelial biopsies	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100/1–24 h	Proteolytic enzyme	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V 300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Kinetic Imaging	51 comets	Tail length	Tisch et al., 2005
Nasal epithelial biopsies	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, 1% triton X-100/1 h	50 mg protease, 10 mg hyaluronidase, 10 mg collagenase in 10 ml Ham's F12	1 mM EDTA, 300 mM NaOH	60 min/20 min, 0.8 V/cm	0.4 M Tris - HCl, pH 7.4	Ethidium bromide	Komet 3.0 Kinetic Imaging	100 comets	Olive tail moment	Hölzer et al., 2008
Nasal epithelial biopsies	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/1.5 h	100 µl of enzyme mix containing 0.1 g protease, 1 mg DNAse dissolved in 10 ml phosphate buffered saline were prepared in 9 ml Airway epithelial growth medium	200 mM Na_2EDTA, 5 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Trizma base, pH 7.5	Ethidium bromide	Komet 5.5 Kinetic Imaging	100 comets	Olive tail moment Tail length Tail DNA	Ginzkey et al., 2012

(Continued)

Table 5 | Continued

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/1 h	Collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml), pronase E type XIV from Streptomyces griseus (5 mg/ml) dissolved in Bronchial Epithelal Basal Medium	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris pH 7.5	Ethidium bromide	Komet 3.1 Kinetic Imaging	80 comets	Olive Tail moment	Kleinsasser et al., 2001
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/1 h	Collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml), pronase E type XIV from Streptomyces griseus (5 mg/ml) dissolved in Bronchial Epithelal Basal Medium	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris pH 7.5	Ethidium bromide	Komet 3.1 Kinetic Imaging	80 comets	Olive Tail moment	Kleinsasser et al., 2004
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/90 min	Collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml), pronase E type XIV from Streptomyces griseus (5 mg/ml) dissolved in Airway Epithelial Cell Growth Medium	200 mM Na_2EDTA, 10 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Komet 4.0 Kinetic Imaging	80 comets	Olive Tail moment	Sassen et al., 2005

(Continued)

Table 5 | Continued

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma Base, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO/ 90 min	Collagenase P (1 mg/ml), hyaluronidase (1 mg/ml), protease (5 mg/ml) dissolved in Bronchial Epithelal Basal Medium	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA, 1 V/cm	0.4 M Trizma Base, pH 7.5	Ethidium bromide	Komet 3.1 Kinetic Imaging	80 comets	Olive Tail moment	Buehrlen et al., 2007
3D Mini organ-cultures of human inferior turbínate epithelia	DMSO 10 ml, 1 ml Triton X-100, 89 ml alkaline lysis (NaCl 0.9%, Na_2EDTA, Trizma base, N-laurylsarcosin sodium salt)/1 h	Collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml), pronase E type XIV from Streptomyces griseus (5 mg/ml) dissolved in Bronchial Epithelal Basal Medium	200 mM Na_2EDTA, 10 mM NaOH	20 min/20 min, 300 mA, 0.8 V/cm	0.4 M Trizma Base, pH 7.5	Ethidium bromide	Kinetic Imaging	40 comets	Olive Tail moment	Reiter et al., 2009
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma Base, 1% Na sarcosinate pH 10, 1% triton X-100, 10% DMSO	Collagenase P (1 mg/ml), hyaluronidase (1 mg/ml), protease (5 mg/ml) FPG	1 mM Na_2EDTA, 300 mM NaOH	20 min/20 min, 25 V/300 mA, 1 V/cm	0.4 M Trizma Base, pH 7.5	Ethidium bromide	Komet Kinetic Imaging	80 comets	Olive tail moment	Baumeister et al., 2009a
3D Mini organ-cultures of human inferior turbínate epithelia		Collagenase P (1 mg/ml), hyaluronidase (1 mg/ml), protease (5 mg/ml) FPG	-	-	-	Ethidium bromide	Komet 3.1 Kinetic Imaging	80 comets	Olive tail moment	Baumeister et al., 2009b
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% triton X-100, 10% DMSO/1.5 h	Collagenase P (1 mg/ml), hyaluronidase (1 mg/ml), protease (5 mg/ml) FPG	200 mM Na_2EDTA, 5 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Trizma Base, pH 7.5	Ethidium bromide	Komet 4.0 Kinetic Imaging	80 comets	Olive tail moment Tail length	Koehler et al., 2010

(Continued)

Table 5 | Continued

Biomatrix	Lysis solution/Duration	Enzyme digestion	Electrophoresis buffer	Unwinding/ Electrophoresis duration and V/cm	Neutralization buffer	Staining	Visualization and analysis of images	Number of comets	Measurement	References
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO/1.5 h	9 ml Minimum Essential Medium, 0.1 mg/ml protease XIV, 1 mg/ml DNAse DN25, 0.05 mg/ml gentamycin, 100 U/ml penicillin with 1 μg/ml streptomycin, 0.250 U/ml amphotericin B and 2 mM L-glutamine	200 mM Na_2EDTA, 5 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris, pH 7.5	Ethidium bromide	Komet 5.0 Kinetic Imaging	100 comets	Olive tail moment Tail length Tail DNA	Hackenberg et al., 2010
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO/1.5 h	9 ml Minimum Essential Medium, 0.1 mg/ml protease XIV, 1 mg/ml DNAse DN25, 0.05 mg/ml gentamycin, 100 U/ml penicillin with 1 μg/ml streptomycin, 0.250 U/ml amphotericin B and 2 mM L-glutamine	200 mM Na_2EDTA, 5 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Tris pH 7.5	Ethidium bromide	Komet 5.5 Kinetic Imaging	100 comets	Olive tail moment Tail length Tail DNA	Hackenberg et al., 2011
3D Mini organ-cultures of human inferior turbínate epithelia	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10, 1% Triton X-100, 10% DMSO/1.5 h	Collagenase P (1 mg/ml), hyaluronidase (1 mg/ml), protease (5 mg/ml)	200 mM Na_2EDTA, 5 mM NaOH	20 min/20 min, 25 V/300 mA	0.4 M Trizma base, pH 7.5	Ethidium bromide	Komet 5.5 Kinetic Imaging	100 comets	Olive tail moment Tail length Tail DNA	Koehler et al., 2013

pesticides and found that permethrin, DEET, diazinon, pentachlorophenol, lindane, transfluthrin, cyfluthrin and pyrethrum induced DNA damage in nasal epithelial cells; these authors performed enzymatic digestion prior to conducting the comet assay. Hölzer et al. (Ginzkey et al., 2012)compared the genotoxic potential of various chemicals (N-nitrosodiethanolamine, epichlorohydrin, 1,2-epoxibutane, ethylene dibromide, and 1,2-dibromo-3-chloropropane)in nasal epithelial cells that had been derived from rats and humans, and found that human cells were less sensitive than rat mucosal cells to the genotoxic activities of N-nitrosodiethanolamine, ethylene dibromide, and 1,2-dibromo-3-chloropropane, while similar levels of DNA damage induction were observed for epichlorohydrin and 1,2-epoxybutane. Ginzkey et al. (Kleinsasser et al., 2001) found that nicotine increased DNA damage, which was prevented by NAC and mecamylamine.

The comet assay results for *ex-vivo* MOC exposure were primarily generated by the Kleinsasser group, which used the assay to determine chemically induced DNA damage (Kleinsasser et al., 2004; Sassen et al., 2005; Buehrlen et al., 2007; Reiter et al., 2009; Hackenberg et al., 2010, 2011; Koehler et al., 2013). The chemicals tested included: N-nitrosodiethylamine, sodium dichromate, N-methyl-N-nitro-N-nitrosoguanidine, mono (2-ethylhexyl) phthalate, benzo[a]pyrene-7,8-diol-9,10-epoxyde, nicotine, nitrogen dioxide, zinc oxide, and titanium dioxide nanoparticles. DNA damage was induced by all of these chemicals, with the exception of titanium dioxide nanoparticles. The Harreus group also examined MOCs, in which they studied the chemopreventive activity of several compound following oxidative challenge with H_2O_2 or dexamethasone (Baumeister et al., 2009a,b; Koehler et al., 2010); these authors found that NAC, α-tocopherol, quercetin, coenzyme Q10, ascorbic acid, and zinc reduced DNA damage in nasal epithelial cells.

DISCUSSION

Application of the comet assay to determine genotoxicity in nasal epithelial cells does not require modifications to the cell lysis and electrophoresis steps of the protocol that was outlined by Singh et al. (1988). The most important point in the use of nasal epithelial cells is the sampling method. A consensus in the sampling methods used in the three previously mentioned variations was reached. First, cells obtained using a cytobrush are only required to be maintained in cold medium and processed as soon as possible. Second, the most important observation is that the procedure can be considered to be a non-invasive procedure. Biopsies that were used to establish primary cultures required enzymatic digestion before they could be used in the comet assay. Until now, these types of studies have been published periodically; however, only five studies have determined that the nasal epithelium serves as a superior biomatrix to other cell types when assessing DNA damage that is induced by inhaled chemicals. In addition, a consensus was achieved in the sampling procedure when working with 3D miniorgan-cultures, because all of the studies reported the use of enzymatic digestion via solutions with similar compositions by altering the physiological solution to dissolve the enzymes (Kleinsasser et al., 2004; Sassen et al., 2005; Buehrlen et al., 2007; Baumeister et al., 2009a,b; Reiter et al., 2009; Hackenberg et al., 2010, 2011; Koehler et al., 2010, 2013). The congruence in the procedure resulted from the fact that only two groups applied this biomatrix: The Kleinsasser (Kleinsasser et al., 2004; Sassen et al., 2005; Buehrlen et al., 2007; Reiter et al., 2009; Hackenberg et al., 2010, 2011; Koehler et al., 2013) and Harreus groups (Baumeister et al., 2009a,b; Koehler et al., 2010). The homogeneity between the procedures validated the assay because comparison of basal DNA damage reflected low variability; therefore, it may be important for new studies to employ this cell type and apply the procedure that was previously established by these groups.

GUIDELINES

It is obvious that generate a unified protocol for all kind of epithelial cells is impossible. However, this section provides a general comet assay procedure for *ex-vivo* and *in-vivo* epithelium samples.

In the present paper, we mention the advantages and shortcomings of the use of alternative biomatrices to assess DNA damage in human populations, focusing on the methodological characteristics of each type of epithelium and taking the sampling protocol, pre-processing, and post-sampling storage into consideration, as well as the possibilities of sample (snap) freezing and the need to adapt the classical alkaline comet protocol. The advantages to use epithelial cells to mapping DNA damage by comet assay is the possibility to obtain samples with non-invasive methodologies for *in-vivo* studies in a safety and cheapest way. Epitheliums are in direct contact with xenobiotics and endogenous damage inductors, being an attractive biomatrice to evaluate individual genotoxicity to several compounds in the case of 3D miniorgans establish by nasal epithelium. Their applicability in clinical diagnostic confers a potential use in patients across time. Some disadvantages to take in account are the invasive procedures for *ex-vivo* studies, the expensive cost to sampling just to determine DNA damage; however is a perfect possibility to realize multidisciplinary studies when the invasive procedure is required.

The general guideline to realize comet assay in epithelial cells require the correct sampling procedure, to follow the alkaline version proposed by Singh et al. (1988). Sampling differ between *ex-vivo* and *in-vivo* procedures, in this sense we porpoise protocols to specific epithelium source in early sections (Sections Sampling Protocol and Sample Storage, Comet Assay Sample Preparation for lens; Sampling Protocol and Sample Storage, Comet Assay Sample Preparation for corneal; Sampling Protocol and Sample Storage, Comet Assay Sample Preparation for tear duct; Sampling Protocol and Sample Storage, Comet Assay Sample Preparation for buccal; Sampling Protocol and Sample Storage, Comet Assay Sample Preparation for nasal cells).

LENS EPITHELIAL CELLS

Slide preparation. Pre-coated with 0.75% NMPA as fist layer, second layer with 1% LMPA mixed with cell suspension.

Lysis solution. The original recipe (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, in fresh add 1% triton X-100, 10% DMSO) during overnight incubation.

Electrophoresis solution. The original recipe (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13).

Unwinding and electrophoresis. Incubation periods of 20 min, close to 300 mA.

Neutralization solution. The original recipe (0.4 M Tris pH 7.5* for EtBr stain*).

Number of nucleoids evaluated. 50 comets.

CORNEAL CELLS

Slide preparation. Pre-coated with 1% NMPA as fist layer, second layer with 1% LMPA mixed with cell suspension.

Lysis solution. The original recipe (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, in fresh add 1% triton X-100, 10% DMSO) during overnight incubation.

Electrophoresis solution. The original recipe (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13).

Unwinding and electrophoresis. Incubation periods of 20 min, close to 300 mA.

Neutralization solution. PBS, H_2O** (For SYBR stain**).

Number of nucleoids evaluated. 50 comets.

TEAR DUCT CELLS

Slide preparation. Pre-coated with 0.5% NMPA as fist layer, second layer with 0.5% LMPA mixed with cell suspension, and third layer with 0.5% LMPA.

Lysis solution. The original recipe (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, in fresh add 1% triton X-100, 10% DMSO) during 1 h incubation.

Electrophoresis solution. The original recipe (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13).

Unwinding and electrophoresis. Incubation periods of 20 min, close to 300 mA.

Neutralization solution. The original recipe (0.4 M Tris pH 7.5* for EtBr stain*).

Number of nucleoids evaluated. 50 comets.

BUCCAL EPITHELIAL CELLS

Slide preparation. Pre-coated with 0.5% NMPA as fist layer, second layer with 0.5% LMPA mixed with cell suspension, and third layer with 0.5% LMPA.

Lysis solution 1. 2.5 M NaCl, 100 mM Na_4 EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, in fresh add 1% triton X-100, 10% DMSO, during 1 h incubation.

Lysis solution 2. Fresh solution (2.5 M NaCl, 100 mM Na_4 EDTA, 10 mM Tris HCL, 1% Na sarcosinate pH 10, in fresh add 1% triton X-100, 10% DMSO) add Proteinase K (1 mg/ml) warm to 37°C and 1 h incubation.

Electrophoresis solution. The original recipe (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13).

Unwinding and electrophoresis. Incubation periods of 20 min, close to 300 mA.

Neutralization solution. The original recipe (0.4 M Tris pH 7.5* for EtBr stain*).

Number of nucleoids evaluated. 50 comets.

NASAL CELLS

Slide preparation. Pre-coated with 0.5% NMPA as fist layer, second layer with 0.5% LMPA mixed with cell suspension, and third layer with 0.5% LMPA.

Lysis solution. The original recipe (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, in fresh add 1% triton X-100, 10% DMSO) during 1 h incubation.

Electrophoresis solution. The original recipe (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13).

Unwinding and electrophoresis. Incubation periods of 20 min, close to 300 mA.

Neutralization solution. The original recipe (0.4 M Tris pH 7.5* for EtBr stain*).

Number of nucleoids evaluated. 50 comets.

Our suggestion is follow the comet assay procedure in a close way to the Singh et al. (Singh et al., 1988) protocol to diminish the variability between groups. In addition, is important consider that buccal epithelial cells is the unique cell type that require lysis enrichment with proteinase K to obtain free nucleosomes as part of the comet assay protocol.

There are different modifications that needs to be imporved during sampling to obtain a cellular suspension friendly to comet assay or primary culture stablishment.

CONCLUSIONS

Over a 30 year period, the comet assay has been employed in molecular epidemiology as a robust biomarker of the early effects of diseases on human populations. Over the past 10 years in particular, the alkaline assay has been shown to play an important role in monitoring the effects of occupational and environmental hazards. The applicability of the comet assay to almost any cell type confers the important advantage of exploring the use of other biomatrices, such as epithelial cells.

Epithelia are sheets of cells that either line the walls of cavities and channels or, in the case of skin, serve as the outside covering of the body. By the first decades of 20th century, detailed histological analyses had revealed that normal tissues containing epithelia are all structured similarly (Kruze, 1994). In addition, the possibility of obtaining epithelial cells using biopsies or less invasive procedures was the perfect match for applying the comet assay to evaluate DNA damage.

The studies reviewed in the present manuscript can be clearly divided into one of two groups: Those with clear clinical applications (lens and corneal epithelial cells) and those examining the use of epithelial cells as biomarkers for genotoxicity assessments in human monitoring and under *in vitro* conditions.

In the first group, lens cells have been shown to be a useful tool for DNA damage detection in individuals with cataracts. This pathology primarily results from oxidative stress and UV radiation, with these cells producing opacity and developing genotoxicity that can be detected using the alkaline comet assay. These factors suggest that the comet assay may be applied to understand other eye pathologies, such as macular degeneration. Corneal cells also fall in to this group and have been used with the aim of determining DNA damage in cells with the potential to be transplanted, although additional damage may be induced by the manipulation. With respect to both of these cell types, few studies have been conducted (**Tables 1**, **2**). However, the studies that have been conducted suggest the feasibility of their use in toxicology, pharmacy, regenerative medicine, and tissue culture.

The group in which epithelial cells were used as genotoxicity biomarkers in human monitoring involves studies using tear duct, buccal, and nasal epithelial cells (**Tables 3–5**). A tear duct study determined genotoxicity in humans that had been exposed

to air pollution, which generated ophthalmological symptomatology. Therefore, exfoliated eye cells may be a sensitive target for the genotoxic evaluation of ophthalmological products, cosmetics, and gasses that may come in direct contact with the eye. The use of buccal epithelial cells in the comet assay has versatility in determining genotoxicity, such as the use of the micronucleus test. Over the past few years, the use of these types of cells in human monitoring has increased in the field of odontology, evaluating several types of chemicals, and odontological procedures, because buccal epithelial cell renewal occurs every 10–14 days. The nasal epithelial cell renewal rate is approximately once every 30 days, reflecting their utility in detecting DNA damage that is induced by the interaction of several substances or environmental conditions during a recent period of exposure when the comet assay is applied during human monitoring. However, over the past 10 years, the use of nasal epithelial cells has been proposed to establish 3D cultures of these cells and determine genotoxicity using an *in vitro* model.

At present, epithelial cells are not sufficiently utilized for genotoxicity evaluations. An important argument for using epithelial cells is that the majority of human tumors arise from epithelial tissues. Detection of DNA damage in this cell types of cells can be done on single level using the comet assay.

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Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay

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Key Concepts

- The two-dimensional Two-Tailed Comet assay (TT-comet) protocol is a valuable technique to differentiate between single-stranded (SSBs) and double-stranded DNA breaks (DSBs) on the same sperm cell.
- Protein lysis inherent with the TT-comet protocol accounts for differences in sperm protamine composition at a species-specific level to produce reliable visualization of sperm DNA damage.
- Alkaline treatment may break the sugar–phosphate backbone in abasic sites or at sites with deoxyribose damage, transforming these lesions into DNA breaks that are also converted into ssDNA. These lesions are known as Alkali Labile Sites "ALSs."
- DBD–FISH permits the *in situ* visualization of DNA breaks, abasic sites or alkaline-sensitive DNA regions.
- The alkaline comet single assay reveals that all mammalian species display constitutive ALS related with the requirement of the sperm to undergo transient changes in DNA structure linked with chromatin packing.
- Sperm DNA damage is associated with fertilization failure, impaired pre-and post- embryo implantation and poor pregnancy outcome.
- The TT is a valuable tool for identifying SSBs or DSBs in sperm cells with DNA fragmentation and can be therefore used for the purposes of fertility assessment.

Sperm DNA damage is associated with fertilization failure, impaired pre-and post- embryo implantation and poor pregnancy outcome. A series of methodologies to assess DNA damage in spermatozoa have been developed but most are unable to differentiate between single-stranded DNA breaks (SSBs) and double-stranded DNA breaks (DSBs) on the same sperm cell. The two-dimensional Two-Tailed Comet assay (TT-comet) protocol highlighted in this review overcomes this limitation and emphasizes the importance in accounting for the difference in sperm protamine composition at a species-specific level for the appropriate preparation of the assay. The TT-comet is a modification of the original comet assay that uses a two dimensional electrophoresis to allow for the simultaneous evaluation of DSBs and SSBs in mammalian spermatozoa. Here we have compiled a retrospective overview of how the TT-comet assay has been used to investigate the structure and function of sperm DNA across a diverse range of mammalian species (eutheria, metatheria, and prototheria). When conducted as part of the TT-comet assay, we illustrate (a) how the alkaline comet single assay has been used to help understand the constitutive and transient changes in DNA structure associated with chromatin packing, (b) the capacity of the TT-comet to differentiate between the presence of SSBs and DSBs (c) and the possible implications of SSBs or DSBs for the assessment of infertility.

Keywords: Sperm DNA damage, fertility, male factor, mammalian reproduction, eutheria, metatheria, prototheria

INTRODUCTION

Different methodologies exist to detect DNA breaks in somatic and sperm cells. Some of these techniques are based on the propensity of the DNA molecule to form single stranded DNA stretches in the presence of stressing environments such as alkaline or acid solutions. Alkaline sucrose gradient sedimentation, alkaline elution or alkaline DNA precipitation, are biochemical techniques based on alkaline DNA unwinding that have been used to assess the presence of single-strand DNA breaks (SSBs). Variants also exist where the DNA molecule is processed under non-denaturing buffered conditions; under these pH conditions, detection of double-strand DNA breaks (DSBs) is feasible, especially when the DNA molecule is strongly de-proteinized prior to electrophoresis (Ahnstrom, 1988; Olivie, 2006; Olive and Banáth, 2007). DSBs seem to be more relevant to the production of chromosome aberrations and can arise as a consequence of insufficient or inefficient DNA repair activity to restitute the original linear chromosomal DNA continuity; these chromosomal rearrangements or deletions may result in stoppage or delay of the cell cycle, and cell death (Marchetti et al., 2007).

Intercellular heterogeneity in DNA damage production or repair can be assessed *in situ* using morphological procedures such as the single-cell electrophoresis assay commonly known as the comet assay (McKelvey-Martin et al., 1993; Collins, 2004). The comet assay is a straightforward method for assessing DNA strand breaks in eukaryotic cells and the methodology is relatively simple. Basically, live cells can be embedded into a microgel on a microscope slide, lysed with a controlled high salt and detergent solution to form nucleoids which are visible under fluorescence microscopy and which form a comet image after migration of DNA fragments associated with electrophoresis. The intensity of migrated DNA at the comet tail, relative to the head, is a directly linearly related to the quantity of DNA breaks originally present in the DNA molecule (Collins, 2004). The original comet assay can be considered as modification of the "halo" assay as conceived by Cook et al. (1976); the connection between the concept of "halo" and the "comet" emerged from Ostling and Johanson (1984) some 8 years later. The first version of the comet assay was performed under neutral conditions but using relatively low strength protein removal; this is interesting, because under these conditions, the morphology of the comet has been found to be highly dependent on the capacity of the protein depletory agents to induce chromatin relaxation of a supercoiled DNA molecule. A new modification of the original neutral comet assay, as conceived by Ostling and Johanson (1984), was developed by Singh et al. (1988), but in this case, electrophoresis was performed under an alkaline-DNA denaturant environment. The rationale of this methodology was to mobilize single stranded DNA molecules unwound from the end of the breaks. Discrepancies exist in the literature as to what is the "real" information derived from the different assays in terms of DNA break production (SSBs or DSBs) because the scenarios for which the techniques have been used (see examples in Collins, 2004) are as different as the chromatin organization of the cells subjected to analysis. It is not surprising that the behavior of somatic cell and gametic chromatin to equivalent treatments varies so dramatically when you consider the different levels of tissue dependent heterochromatinization, the highly histonized nature of somatic chromatin and the genetic inactivity and histone replacement by protamines during spermatogenesis.

THE TWO-TAILED (TT) COMET ASSAY—THE IMPORTANCE OF SPECIES-SPECIFIC PROTEIN LYSIS

The possibility exists of combining non-denaturing and denaturant conditions to the same sperm nucleoid. In this case, the species-specific de-proteinized sperm is first subjected to an electrophoretic field under non-denaturing conditions to mobilize isolated free discrete DNA fragments produced from DSBs; this is then followed by a second electrophoresis running perpendicular to first one but under alkaline unwinding conditions to produce DNA denaturation exposing SSBs on the same linear DNA chain or DNA fragments flanked by DSBs. This procedure results in a two dimensional comet tail emerging from the core where two types of original DNA affected molecule can be simultaneously discriminated. The two-dimensional perpendicular tail comet assay (TT-comet) is an excellent methodological approach to distinguish between single and double strand DNA damage within the same cell. In this review, we present TT-comet assay data that our group has published for the three sub-classes of mammals, the prototheria (echidna), metatheria (koala and kangaroo) and eutheria (Enciso et al., 2009; Johnston et al., 2009; Enciso et al., 2011a,b; Portas et al., 2009; Zee et al., 2009; Gosálvez et al., 2014).

The difference in sperm chromatin structure is particularly fascinating as each group has a different protamine amino-acidic composition (**Table 1**) (Vilfan et al., 2004) so that lysing solutions used in the preparation of the TT-comet in order to produce a controlled protein depletion need to be targeted and species-specific to make the analyses comparable. There are two major amino acid residues in protamines that appear to be important for understanding DNA and protein assembly associated with sperm chromatin compactness; these include (a) the presence of cysteine residues that allow the formation of intra- and inter-disulphide bonds and (b) the existence of arginine residues that permit more intense positive or negative charged protamines to interact with the sperm DNA. Species differences in protamine sequences are illustrated in **Table 1**. The relative composition and location of these particular residues in the sperm DNA of the different mammalian taxa combined with their respective relationship to the interspecific heterogeneity of protamine 1/protamine 2 ratio, and the arrested substitution of protamine 1 by protamine 2 (e.g., boar and bull), highlights the uniqueness of sperm DNA molecule when compared to the rest of the soma (Biegeleisen, 2006; Balhorn, 2007; Gosálvez et al., 2011). This phenomenon also makes the comparative investigation of sperm DNA from species other than human or domestic animals experimental model for understanding DNA packaging and fragmentation.

Large structural differences exist between somatic cells and spermatozoa. For example, the replacement of histones by protamines in the sperm cell facilitate; (a) the efficient chromatin packaging to provide additional protection of the DNA during the long journey in the female reproductive tract and (b) the production of an ATP driven flagellum for autonomous displacement capacity. In the majority of mammalian species, especially in eutherian mammals, cysteine residues are present in the

Table 1 | Multiple amino-acid sequence alignment in different mammalian species to show species-specific differences in cysteine residues and arginine content in Protamine 1.

Species	English name	Amino acid reference and alignment	NR	C	A
Homo sapiens	Human	MARYRC CRSQSRSRYY RQRQRS RRRR RRSCQTRRRAMRCCR PRYRPRCRRH	51	6/2	24
Pan troglodytes	Chimpanzee	MARYRC CRSQSRSRCY RQRQRS RRRK RQSCQTQRRAMRCCR RRSRMRRRRH	51	6/2	23
Orictolagus cuniculus	Rabbit	MVRYRC CRSQSRSRCR RRRRR CRRR RRRCCQRRRVRKCCR RTYTLRCRRY	50	9/3	26
Felis catus	Cat	MARYRC CRSHSRSRCR RRRRR CRRR RRRCC RRPRKRVCSR RYRVGRCRRR	50	8/2	28
Ursus arctos	Bear	MARYRC CRSHSRSRCR RRRRR CRRR RRRCCRRRRRRVCCR RYTVVRCRRR	50	9/3	29
Camelus bactrianus	Camel	MARYRC CRSHSRSRCR PRRRR CRRR RRRCCRRRRRRVCCR RYTIIRCRRR	50	9/3	28
Sus scrofa	Boar	MARYRC CRSHSRSRCR PRRRR CRRR RRRCCPRRRRAVCCR RYTVIRCRRC	50	10/3	25
Orcinus orca	Killer Whale	MARNR CRSPSQSRCR RPRRR CRRR IRCC RRQRRVCCR RYTTTRCARQ	47	8/2	21
Equus asinus	Donkey	MARYRC CRSQSQSRCR RRRRRR CRRR RRRC VRRRRVCCR RYTVLRCRRRR	50	8/1	28
Equus caballus	Stallion	MARYRC CRSQSQSRCR RRRRRR CRRR RRRS VRQRRVCCR RYTVLRCRRRR	50	7/2	27
Bos taurus	Bull	MARYRC CLTHSGSRCR RRRRRR CRRR RRRSGRRRRRRVCCR RYTVIRCTRQ	51	7/2	26
Alces alces	Moose	MARYRC CLTHSRSRCR RRRRRR CRRR RRRFGRRRRRRVSCR RYTVIRCTR	50	6/2	27
Capra hircus	Goat	MARYRC CLTHSRSRCR RRRRRR CRRR RRRFGRRRRRRVCCR RYTVVRCTRQ	51	7/2	27
Gazella dorcas	Gazelle	MARYRC CLTHSRSRCR RRRRRR CHRR RRRFGRRRRRRVCCR RYTVVRCTRQ	51	7/2	26
Ovis aries	Ram	MARYRC CLTHSRSRCR RRRRRR CRRR RRRFGRRRRRRVCCR RYTVVRCTRQ	51	7/2	27
Ratus norvergicus	Rat	MARYRC CRSKSRSRCR RRRRR CRRR RRRCCRRRR-RRCCRR RRSYTFRCKRY	51	9/3	29
Mus musculus	Mouse	MARYRC CRSKSRSRCR RRRRR CRRR RRRCCRRRR RRCCRR RSYTIRCKKY	51	9/3	28
Loxodonta africana	Elephant	MARYRC CRSRSRSRCRSRRRRRS HRRR RRCARRRRRTRRGCR RRYSLRRRRY	52	5/1	31
Phascolarctos cinereus	Koala	MARYRH SRSRSRSRYQRRRRRRSRYRSQRRRYRRRRGSRRRRRRGRRRG YRRRYSRRRRY	60	0	37
Planigale maculate sinualis	Planigale*	MARCRRHSRSRSRSRNQCQRRRR RRYNRRR TYRRSRRHSRRRRGRRRGCSRRRYSRRGRRRY	62	3	35
Planigale maculate muculata	Planigale*		63	0	37
Sminthopsis crassicaudata	Dunnart	MARYRRHSRSRSRSRYRRRRRRRS RHHNRRR YRRSRRHSRRRRGRR RGYSRRRYSRRGRRRY			
Didelphis virginiana	Opossum	MARYRRRSRSRSRSRYG RRRR RSRSRR RRSRRR RRRRGRRGRGYHRRSPHRRRRRRRR	58	0	38
Tachyglossus aculeatus	Echidna	MARFRP SRSRSRSLYRRRRRSRR QRSRRGGRQTGPRKITRRGRGRGKSRRRRGRR SMRSSRRRRRRRRN	69	0	36

*NR, Number of residues; C, Cysteine; A, Arginine; *Subspecies of the Common Planigale.*

protamines to create a more condense and well-packed chromatin fiber, so that when the comet assay is performed under either neutral or alkaline conditions, there is a requirement to first pretreat the chromatin to loosen this protective protein by means of a reducing agent (dithiothreitol or beta-mercaptoethanol). This treatment specifically reduces the covalent disulphide (SS) bridges present at both the intra-protamine and inter-protamine molecular level (Bedford and Calvin, 1974a; Yanagimachi, 1994; Vilfan et al., 2004), so that any putative free DNA fragments can be mobilized under an electrophoretic field. An understanding of the inherent peculiarities of sperm DNA structure between the different species of mammals is of fundamental importance when establishing sperm comet assays in novel taxa, so that the technique needs to be appropriately validated for each species in order to account for differences in chromatin structure.

SPERM DNA COMET ASSAY UNDER NON-DENATURING NEUTRAL CONDITIONS

When lysed spermatozoa with no DNA fragmentation are subjected to an electrophoresis under non-denaturing conditions, no substantial comet tails are formed (sperm labeled as normal in **Figure 1A**). In contrast, damaged sperm DNA show extensive migration of DNA fragments from the original sperm core (**Figure 1A**) and these migrating DNA fragments are most likely to be associated with DSBs which were present at the origin; however, one needs to be cautious about this interpretation as these DNA fragments are also likely to contain "internal" single strand breaks that cannot be differentiated. Similar comets can be produced after incubation with classic double strand DNA cutters such as restriction endonucleases. Alu I, for example, is an enzyme that is able to selectively identify and cleave CGTT sites on fixed chromatin (Mezzanotte et al., 1983; Miller et al., 1983) producing substantial DNA release. Restriction endonuclease gives rise to specific DSBs and the extensive production of comets, which is the direct consequence of DNA cleavage produced by enzymatic treatment (Brooks, 1987).

SPERM DNA COMET UNDER ALKALINE DENATURING CONDITIONS

DETERMINATION OF STRUCTURAL COMETS AND ALKALI LABILE SITES IN SPERMATOZOA

DNA breaks are starting points for alkaline DNA unwinding due to the disruption of hydrogen bonds among purines and pyrimidines. Moreover, mutagens may induce DNA base loss and deoxyribose lesions that may be transformed into SSBs by alkaline conditions, being designated as alkali labile sites (ALS). Remarkably, when the spermatozoa of all mammalian species so far analyzed are subjected to denaturant alkaline conditions and electrophoresed, they exhibit a prominent comet tail (Singh and Stephens, 1998; Fernández et al., 2000; Cortés-Gutiérrez et al., 2009) (**Figure 2**). These structural comets are present in the sperm cells of all three different sub-classes of

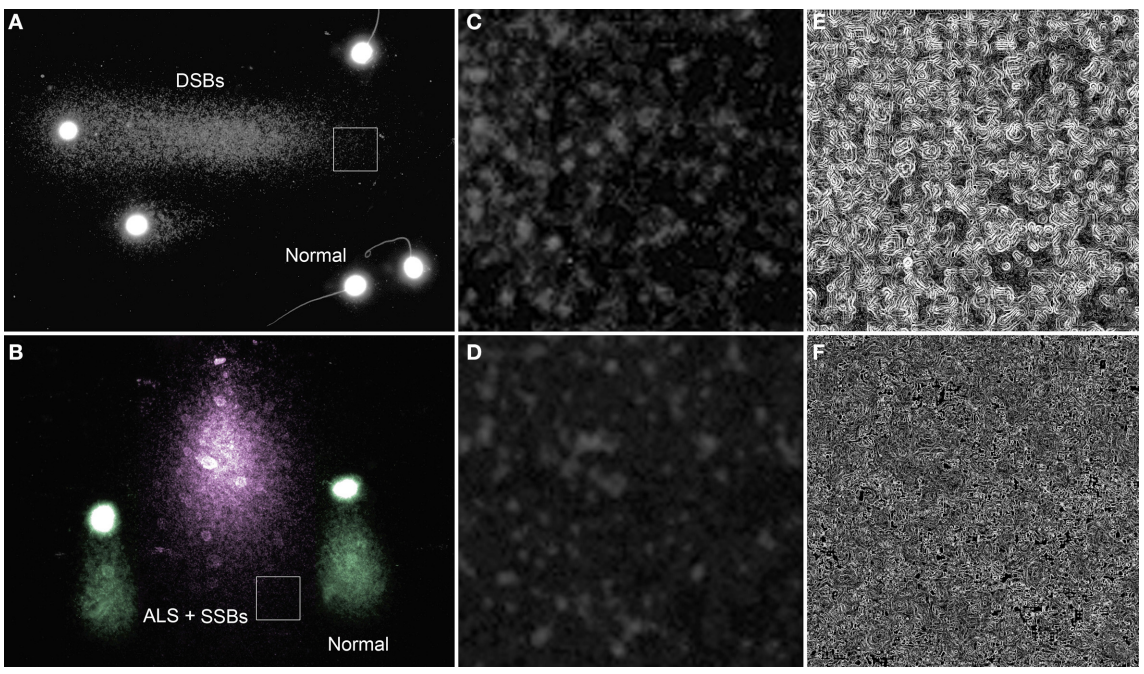

FIGURE 1 | Single neutral and alkaline comets. (A) Neutral comet from rhinoceros *(Rhinoceros sondaicus)* sperm showing a tail of mobilized DNA fragments (right direction of the image) as a consequence of double strand DNA breaks (DSBs) at the origin of the sperm head. Sperm without comet tail (labeled as normal for this condition) do not contain detectable levels of DSBs, but display a small halo of compact chromatin. **(B)** Alkaline comet of rhinoceros sperm (pink comet) showing a tail of mobilized DNA fragments which are interpreted as single strand DNA stretches derived from short DSBs susceptible to be denatured, single breaks (SSBs) and alkali labile associated with structural comets (pseudo-colored green). Magnified regions (box) within the neutral **(C)** and an alkaline comet tail **(D)** are provided to visualize the difference in the chromatin structure along with filtered images to enhance differences in chromatin texture (**E**—neutral and **F**—alkaline).

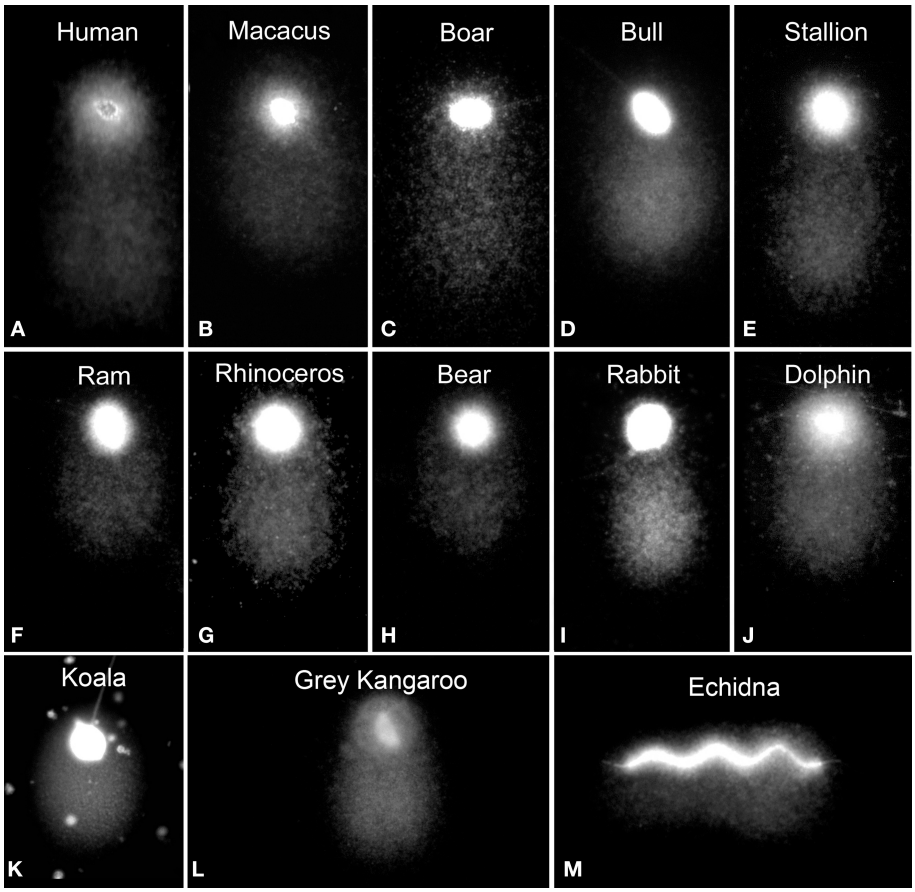

FIGURE 2 | Structural alkaline comets in different mammalian sperm (A–J) eutherian species, (K,L) metatherian species and (M) a prototherian species.

mammals (**Figure 2**). Differences in the length of the tails are observed when different eutherian species are compared; for example, compare human, macacus (*Macacus rehesus*), boar, bull, stallion, ram, rhinoceros, bear, rabbit, dolphin (*Delphinus delphis*) (**Figures 2A–J** respectively); these comer tails appear to be comparatively shorter in the spermatozoa of metharian species subjected to the same experimental conditions; koala and gray kangaroo (*Macropus fuliginosus*) (**Figures 2K,L**, respectively) and in the case of echidna (prototheria), which possesses a unique elongated filiform sperm nucleus, with the comet being observed along the length of the sperm head (**Figure 2M**). Mammalian sperm chromatin appears especially susceptible to alkaline "breakage" and/or denaturation, representing the presence of a high density of SSBs or ALS. Comets after DNA denaturation show a more diffuse chromatin and the visualized DNA fragments at the end of the tail are not as defined as those produced after neutral comets [compare **Figures 1C,E** (neutral) with **Figures 1D,F** (alkaline)]. The presence of a comet tail associated with SSBs or ALSs is not related to any harmful DNA damage but is a consequence or a feature inherent to the sperm chromatin structure; we refer to these comets as "structural" or "constitutive" comets.

Alkali labile sites can also be detected using the DNA Breakage Detection-Fluorescence *In Situ* Hybridization (DBD-FISH) procedure (Fernández et al., 2000; Fernández and Gosálvez, 2002). Using this procedure, cells embedded within an inert agarose matrix on a slide are lysed and the resultant nucleoids exposed to a controlled alkaline denaturation step. Under these conditions, putative DNA breaks are transformed into restricted single-stranded DNA motifs, initiated from the ends of the DNA breaks that may be detected by hybridization using either whole genome or specific fluorescent DNA probes. The specific DNA probe selects the chromatin area to be analyzed. As DNA breaks increase within a specific target, more single-stranded DNA is generated and more DNA probe hybridizes, producing increasing levels of fluorescence (Fernández et al., 2000; Fernández and Gosálvez, 2002) (**Figure 3**). It is noteworthy that when a whole-genome DNA probe is hybridized to somatic cells, the background DBD-FISH signal is not homogeneous and certain chromatin regions are selectively and strongly labeled; this is especially evident when high levels of alkali denaturation are used on the native sperm chromatin (**Figure 3**, DBD-FISH High). It is of interest to highlight that the DNA sequences related with constitutive ALS mostly correspond with certain specific highly repetitive DNA sequences (Fernández et al., 2001; Rivero et al., 2001, 2004). In human leukocytes, the more intense background DBD-FISH areas within the genome correspond to DNA domains containing 5-bp satellite DNA

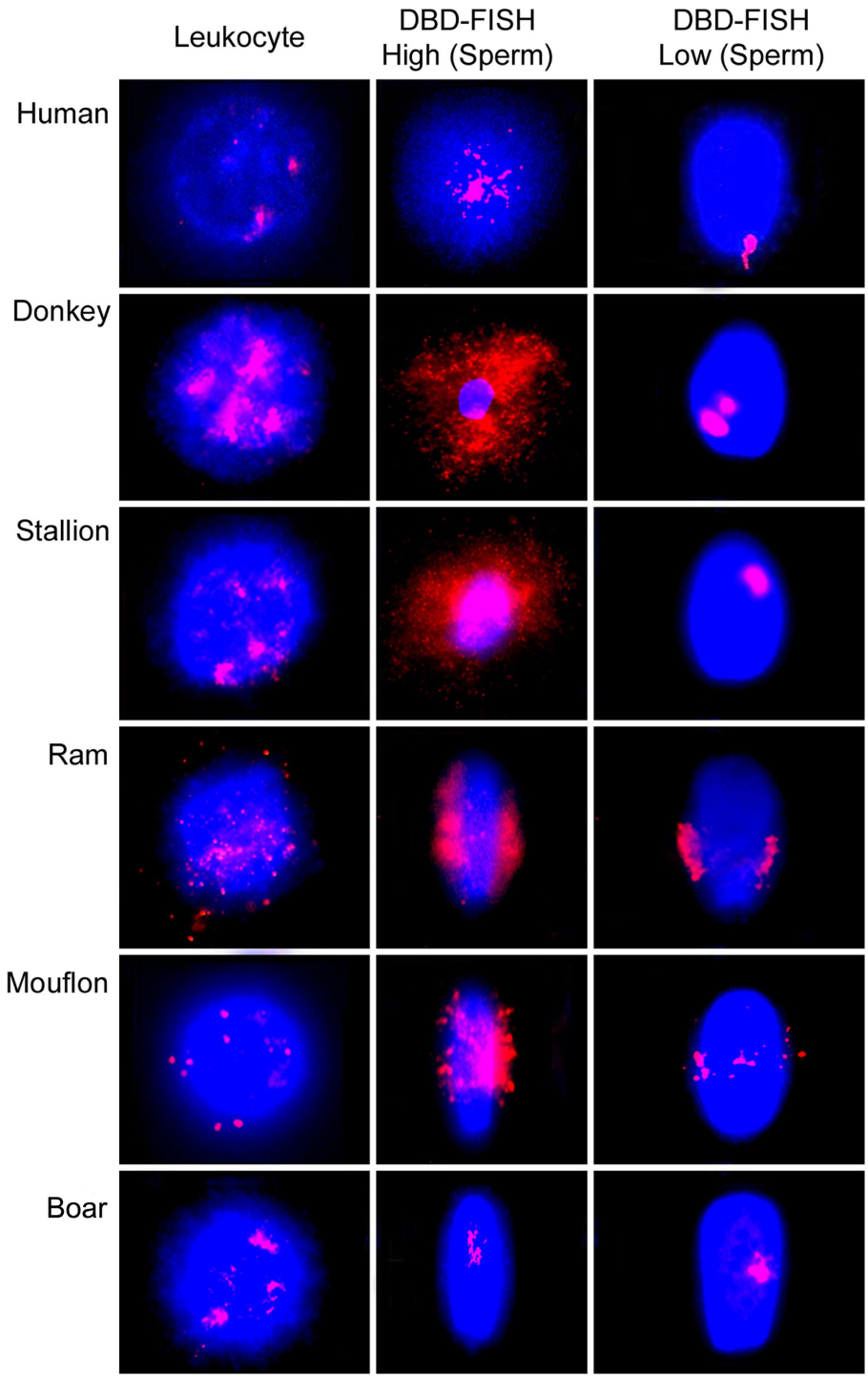

FIGURE 3 | Visualization of the alkali labile sites (ALS) using DNA Breakage Detection-Fluorescence *In Situ* Hybridization (DBD-FISH) as a structural feature of the spermatozoa and peripheral blood leukocytes in different mammal species. High alkali denaturation conditions (DBD-FISH High) show most of the ALS present al the spermatozoa. Mild alkaline conditions (DBD-FISH Low) revealed the localization of regions most sensitive to alkaline denaturation.

sequences (Fernández and Gosálvez, 2002). In mouse splenocytes, the background labeled areas correspond with highly repetitive major DNA satellite sequences located in pericentromeric regions (Rivero et al., 2001), and in Chinese hamster cells, they match to pericentromeric interstitial telomeric-like DNA sequence blocks (Rivero et al., 2004). As indicated, all these native highly alkali-sensitive regions correspond to strongly compacted chromatin domains present in somatic nuclei.

It is also possible to control assay conditions to produce DNA denaturation using very mild alkaline to produce very restrictive single strand DNA stretches to be revealed later by DBD-FISH (See **Figure 3** DBD-FISH-low). Using this approach, it can be demonstrated that the most sensitive genome regions to alkaline denaturation are not randomly localized in the sperm nucleus. Usually there is one or two discrete genome domains consistently localized in each species, but these regional locations differ amongst species. For example, in human spermatozoa, the most alkali sensitive region appears located at the proximal end of the spermatozoon, adjacent to the implantation fossa (Cortés-Gutiérrez et al., 2014a). In other species such as the ram, ALSs are located along the equatorial region of the sperm head and consist of two opposing clusters of hybridization. In the case of the stallion, ALS show a tendency to cluster, but the closely related donkey presents two discrete clusters of hybridized signal. Both signals tended to be localized at the equatorial-distal regions of the sperm. Boar spermatozoa present a discrete signal localized in the central region of sperm (Cortés-Gutiérrez et al., 2009).

The differences in the quantity of ALSs between somatic cells and spermatozoa can also be evidenced using DBD-FISH (Fernández et al., 2000; Fernández and Gosálvez, 2002; Cortés-Gutiérrez et al., 2009) (**Figure 3**). Using this technique, the hybridization signal produced with a whole-genome DNA probe is 12.7 times more intense in spermatozoa than the signal obtained in peripheral blood leucocytes (Muriel et al., 2004). In mouflon (*Ovis musimon*), the density of ALS in sperm is eight times higher than that of the somatic cells. In sheep, both leucocytes and sperm cells exhibited a large quantity of ALS, being four times more abundant in sperm (Cortés-Gutiérrez et al., 2008). In donkey and stallion, the relative abundance of ALSs was also four times higher in spermatozoa than in somatic cells. ALSs in the sperm of donkey was 1.3 times greater than in stallion and the length of the comet tail obtained in donkey sperm was 1.6 times longer than that observed in horse and the differences were significant ($P < 0.05$) (Cortés-Gutiérrez et al., 2014b). Boar spermatozoa are unique in this respect as ALSs are 12 times higher in their leukocytes compared to spermatozoa (Cortés-Gutiérrez et al., 2008). Interestingly, only the satellite DNA sequences integrated at the pericentromeric heterocromatin of all metacentric chromosomes of the karyotype were contributing to produce ALSs in the boar (Cortés-Gutiérrez et al., 2008). Later it was found that the low quantity of ALS detected after DBD-FISH in the boar was in fact an artifact linked to the limited ability of the alkaline denaturation and protein lysis used for the DBD-FISH procedure and the extremely strong compacted chromatin structure present in the boar sperm cell, and which is dependent on only protamine 1 and which presents with 5 cysteine residues per protamine molecule (Gosálvez et al., 2011).

We propose that structural sperm comet tails can be interpreted as a consequence of the peculiar massive presence of constitutive ALSs in natural chromatin and is likely to be a manifestation of a physical and transient circumstance linked to the specific need for efficient chromatin packing (Allen et al., 1997). These regions also seem to be especially susceptible to *in situ* enzymatic digestion by "mung bean nuclease" so could correspond to stretches of partially denatured single-stranded DNA, which could act as starting points of DNA denaturation by alkaline conditions (Bedford and Calvin, 1974b; Fernández et al., 2000; Cortés-Gutiérrez et al., 2014a). While it has not been fully demonstrated whether these regions correspond to abasic (apurinic or apyrimidinic) sites that can be converted into DNA breaks by the alkali, this possibility certainly exists; in fact, spermatozoa are quite recombinogenic in the presence of exogenous DNA (Sakkas et al., 2002; Fernández-González et al., 2008).

Differences also exist in the length of the structural comets observed among different species with shortest found in the bear and koala (**Figure 2**). This phenomenon has not been studied in detail, although the most parsimonious hypothesis to assume would be that the size of the structural comet is related to the differential susceptibility of the chromatin in each species to an equivalent treatment to produce DNA denaturation; this phenomenon deserves more thorough investigation. Interestingly, within each species, structural comets do not show large differences in comet tail length (**Figure 4A**) but it is possible to detect differences in their respective fluorescence intensity as illustrated in the accompanying profiles (**Figures 4A,B**).

DIFFERENTIATION OF INDUCED MUTAGENIC SSBs FROM CONSTITUTIVE ALS

Under alkaline conditions, structural comet tails associated with ALS can be differentiated from "real" DNA damage by observing the density and length of the comet tail. Sperm nuclei with "real" denatured DNA derived from DSBs, SSBs and ALSs have significantly longer comet tails (pink comet in **Figure 1B**) than those that are merely structural comets (green comets in **Figure 1B**). **Figures 4A,B** show the difference in the DNA density on sperm comets in stallion conducted under alkaline conditions, although the migration distance from the core is quite similar in all the cases. In **Figures 4C,D**, we show the differences in both DNA density in the comet and comet tail length as visualized in koalas. In this case, the difference in the DNA migration between affected and non-affected sperm is prominent because the structural comet in this species is not as large as that observed in other eutherian species. When the sperm is incubated with agents that primarily induce SSBs such as hydrogen peroxide (Yamamoto, 1969) or sodium nitroprusside (a nitric oxide donor) (Lin et al., 2000; Ichikawaa et al., 2008), highly enlarged comet tails emerge from the core following denaturing conditions (Lin et al., 2000). The length of the tail as well as the intensity profile of staining of the DNA migrated from the core is related to the amount of induced damage.

TWO TAIL COMET CONDUCTED UNDER SEQUENTIAL NEUTRAL AND ALKALINE CONDITIONS

Figure 5 shows a TT-comet of a human spermatozoa as visualized under fluorescence microscopy (original image -5A- and electronically filtered -5B-) and the putative distribution of the DNA breakage present in the original spermatozoon. To produce a TT comet, deproteinized sperm are initially subjected to a neutral electrophoresis that results in the mobilization of free DNA-chromatin fragments associated with DSBs along the X-axis. While the DNA domain on the X axis represents DSBs at the origin, the tail may also contain SSBs that could potentially

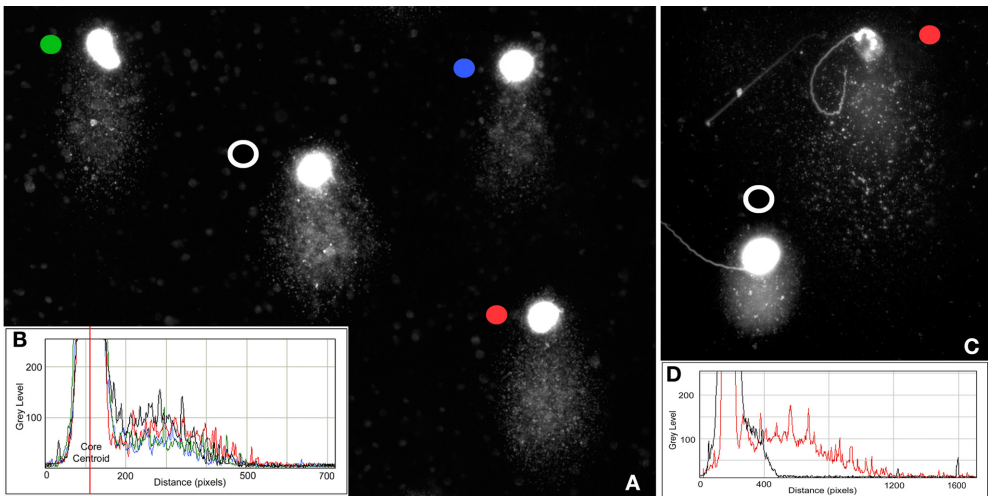

FIGURE 4 | (A) Stallion structural comets showing differences in the density of DNA but similar tail length; **(B)** Density profile of horse comets showing differences in fluorescence intensity—different colored circles correspond to the colored graphic profiles of individual spermatozoa; **(C)** Structural (black dot) and damaged (red dot) koala spermatozoa showing differences in the amount of DNA and DNA migration distance; **(D)** Density profile of koala comets showing differences in fluorescence intensity and length of comet tail.

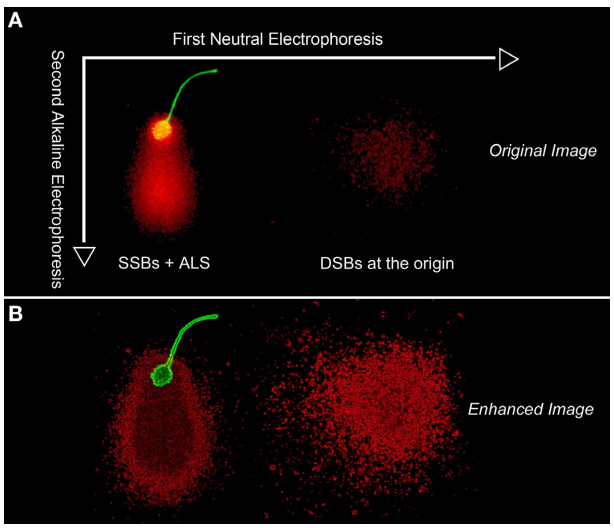

FIGURE 5 | Original (A) and digitally enhanced image (B) to show whole DNA fragment distribution DNA map following a two tailed comet assay in a human sperm cell. The first neutral electrophoresis results in a horizontal migration along the X axis of DNA fragments formed as a consequence of DSBs. After turning the microgel 90°, the second electrophoresis results in the migration of DNA along the vertical Y axis and is conducted under alkaline conditions; the alkaline comet assay reveals both structural Alkali Labile Sites (ALSs) and "true" SSBs that have elongated comet tails. The green fluorescence is associated with proteinaceous remnants of the sperm head and flagellum.

be denatured when exposed to the later alkaline conditions and run in a second electrophoresis at 90° to the first one; under these altered conditions, the DNA fragments would then migrate along the Y axis; this phenomenon is well illustrated in the enhanced image (**Figure 5B**). The comet tail emerging from the sperm nuclear core along the Y-axis contains single stranded DNA stretches, which were produced after denaturing single and double strand breaks existing at the origin that were not displaced during the neutral electrophoresis. Initially, they could be long double-stranded DNA fibers containing DNA nicks at different positions in both strands, but they were too large to be mobilized during the first non-denaturing electrophoresis; however now under alkaline conditions, they can be readily mobilized from the core after DNA denaturation, migrating perpendicularly to the first electrophoretic run. DNA molecules forming the comet tail during the first neutral electrophoresis on the X axis are similarly denatured and displaced along the Y axis; they represent a cloud of single strand DNA stretches emerging from a primary cloud of non-denatured DNA formed from double strand breaks at the origin.

The TT-comet assay has the potential to define four main sperm comets types, which contain different DNA damage at the origin. **Figure 6A** shows the four main TT-comets as observed in the human sperm cell; (1) TT-comet with tails showing a structural comet in the Y axis (**Figure 6A**; yellow comet); (2) TT-comet with ALSs and SSBs with long tails in Y axis (**Figure 6A**; pink comet); (3) TT-comet with both DSBs and SSBs-ALSs tails in the X and Y axis respectively **Figure 6A**; blue comet) and (4) TT-comet with DSBs showing comet tails migrating along the X axis and a structural comet in the Y axis (**Figure 6B**). It is interesting to highlight that the morphology of the DNA fragments on both axes (X-Neutral and Y-Alkaline) have a differently texturized chromatin (Bedford and Calvin, 1974a; Yanagimachi, 1994; Vilfan et al., 2004; Gosálvez et al., 2011).

The tail on the X axis, especially the DNA localized at the closer regions to the core, are visualized as discrete and sharp fluorescent dots, which may be representing typical DNA fragments formed from double-strand breaks at the origin; as indicated in **Figure 1**, these are especially evident in single neutral comets

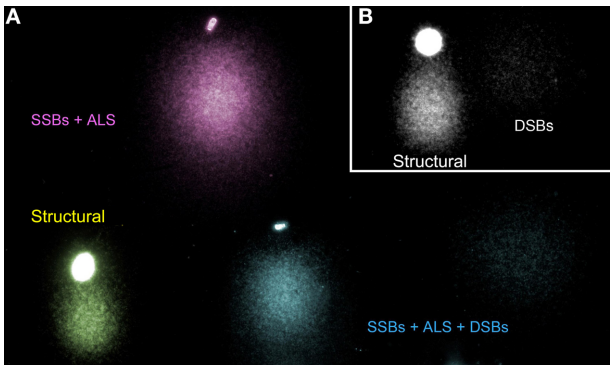

FIGURE 6 | Pseudo-colored images of sperm DNA following a TT comet assay and illustrating different comet morphology associated with different types of DNA damage. (A) Type 1 (yellow)—structural comet; Type 2 (pink)—SSBs plus ALSs; Type 3 (blue) DDBs, SSBs plus ALS within the same TT comet and **(B)** Type 4 (gray)—DSBs and structural comet.

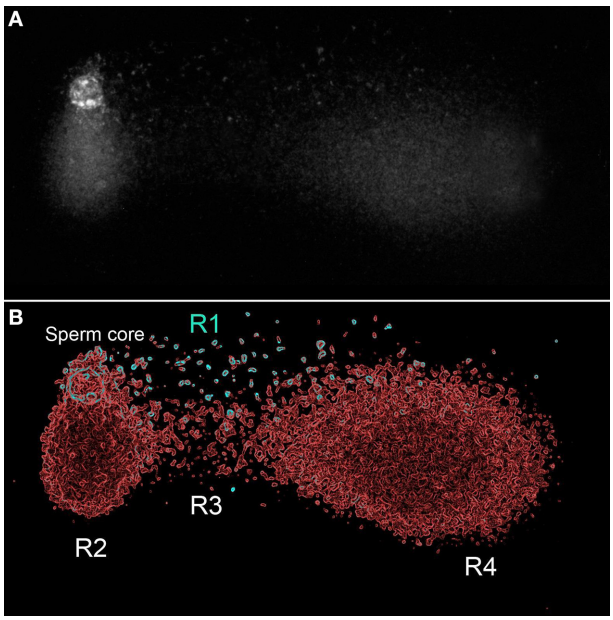

FIGURE 7 | TT comet produced after controlled double strand DNA cleavage using the restriction enzyme Alu-I (AG_CT). Original image **(A)**, and panel electronically enhanced **(B)**. R: different regions identified at the TT-comet. See text for detailed explanation.

(**Figures 1C,E**). The comet tails of the Y axis, which represent entangled single strand DNA motifs, are more compact and the whole tail is "fuzzy" in appearance (**Figures 1B,D,F**).

The tail in the X-axis reflects the DNA fragments (DSBs) mobilized from the core that were denatured after the second electrophoretic run. The tail in the Y axis reflects comet structural in the spermatozoa (**Figure 1A**). To explain the possible distribution of the actual state of the DNA in this comet, we have regionalized the original image in **Figure 7A** in four regions (R1–R4); the results are presented on an electronically enhanced image (**Figure 7B**). R1 includes long DNA fragments (DSBs). DNA fragments of equivalent characteristics but shorter were mobilized under neutral conditions to the end of the comet tail. R2 includes single stranded DNA stretches originating from the constitutive comet at the original spermatozoa. R3 includes short single strand DNA stretches, which are a consequence of the DNA denaturation produced on DNA fragments displaced with the neutral comet and originally positioned at equivalent areas of R1. R4 includes single stranded DNA stretches, which are a consequence of DNA denaturation produced from enzymatic DSBs at the origin and displaced with the neutral comet. Double stranded DNA fragments at R1 located at the end of the comet tail (**Figure 7B**) do not exist, because they were formed by short double stranded DNA fragments that moved with neutral electrophoresis but which, subsequently, were denatured with the second alkaline electrophoresis.

SPERM DNA DAMAGE AND THE DETECTION OF SSBs OR DSBs FOR DIAGNOSTIC VALUE

High integrity of sperm DNA is an obvious requisite for normal embryonic development and a successful pregnancy. Extensive research in human and different animal species indicates that the fraction of sperm cells containing damaged DNA appears to be higher in infertile males than in fertile controls (Castilla et al., 2010). Moreover, males with poor semen quality are more likely to show a higher percentage of sperm with nuclear DNA damage than males with normal semen parameters (Gosálvez et al., 2013). Sperm DNA fragmentation can influence fertilization, embryo quality and development, blastocyst formation, and pregnancy rate; it also may lead to congenital malformations and genetic illnesses, as well as potentially increase the risk of certain cancers in related offspring (Fernández-González et al., 2008). The presence of sperm DNA damage is thought to be linked to three possible mechanisms. The first of these involves abortive apoptosis during meiosis I resulting in ejaculated spermatozoa, which, albeit defective, escape the apoptotic pathway (Sakkas et al., 2002). The second primary mechanism is defective chromatin condensation during spermiogenesis that involves inappropriate protamination and insufficient chromatin packaging. In fact, DNA breaks are produced to eliminate DNA torsional stress when substituting histones by protamines (Marcon and Boissonneault, 2004), so that unrepaired DNA breaks could persist in mature sperm. The third mechanism includes oxidative stress resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant capacity (Agarwal et al., 2003).

The causes of sperm DNA damage resulting in SSBs or DSBs are extremely varied and include exposure to adverse environmental factors such as pesticides, radiation, smoking or pathological situations such as cancer, varicocele and infection. This and presumably other causes lead to the generation of sperm DNA breakage are mediated through one or a combination of the mechanisms identified above. Given knowledge as to the origin of DNA fragmentation in spermatozoa, we might also expect to see different types of DNA lesion that could possibly be predictive or diagnostic in nature. For example, nucleases, either endogenous or exogenous, should produce SSBs and/or DSBs whereas DNA breaks produced by chromatin remodeling during spermiogenesis appear to correspond to DSBs

produced by topoisomerase II (Laberge and Boissonneault, 2005). Finally, ROS and other radical molecules like those derived from nitric oxide should generate mainly SSBs and many different types of DNA base damage, including mutagenic ALSs (Reiter, 2006).

The implication of sperm DNA damage in fertilization and embryo development should depend on the balance between the DNA damage from the sperm and the oocyte's repair capacity. Moreover, the type and/or complexity of DNA lesions in the different sperm can vary and this also must influence the embryonic development. After penetration into the oocyte, sperm with extensive DSBs associated with apoptotic-like processes would exceed the repair capacity of the oocyte, leading to delayed paternal DNA replication, paternal DNA degradation and arrest of embryo development (Gawecka et al., 2013). Conversely, when sperm DNA damage is composed mainly of a low level of DSBs, SSBs, abasic sites, and/or base damages, the oocyte's various specific DNA repair pathways are likely to be effective, so that the DNA of male pronucleus should function normally during syngamy and early embryonic development. Nevertheless, some mis-repaired or unrepaired DNA lesions could still potentially lead to mutations or chromosome aberrations. Unrepaired SSBs or other lesions types may also result in DSBs when DNA is replicating, leading to structural chromosomal abnormalities (Marchetti et al., 2007). If these aberrations are unstable, they are likely to affect the correct mitotic segregation of chromosomes, resulting in genomic instability and cell death, and thereby adversely affect embryo development. When DNA repair is complete, the morula and blastocyst stages can be achieved; the paternal genome should be expressed normally at this stage, so a pregnancy would be more likely. If the repair processes are not totally efficient, blastocyst arrest or spontaneous abortion may result (Fatehi et al., 2006). The differentiation of the types and levels of DNA damage that may coexist in the different sperm, therefore, provide relevant information to the study of male infertility, a technique like TT-comet could be of great value for this purpose.

CONCLUSIONS

(i) When preparing for the application of TT-comet assay it is important to recognize that protamine composition in mammals is species-specific and protein lysis needs to be accordingly validated and adjusted on a species-specific basis.

(ii) The TT-comet assay is an excellent method of discriminating between the presence of single and/or double strand breaks in the DNA in the same sperm cell.

(iii) Structural sperm comets are correlated with the regional presence of alkali labile sites (ALS) which can be mapped using DNA breakage detection coupled with fluorescence *in situ* hybridization (DBD-FISH).

(iv) Structural comets in the normal sperm, as revealed under alkaline DNA denaturing conditions, are a constitutive and transient circumstance, linked to the specific need for efficient chromatin packing. They are present in the sperm of all mammalian species so far analyzed.

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Using a medium-throughput comet assay to evaluate the global DNA methylation status of single cells

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The comet assay is a simple and cost effective technique, commonly used to analyze and quantify DNA damage in individual cells. The versatility of the comet assay allows introduction of various modifications to the basic technique. The difference in the methylation sensitivity of the isoschizomeric restriction enzymes HpaII and MspI are used to demonstrate the ability of the comet assay to measure the global DNA methylation level of individual cells when using cell cultures. In the experiments described here, a medium-throughput comet assay and methylation sensitive comet assay are combined to produce a methylation sensitive medium-throughput comet assay to measure changes in the global DNA methylation pattern in individual cells under various growth conditions.

Keywords: medium-throughput comet assay, global DNA methylation, 5-Aza-dcR, single cells, cytosine extension assay (CEA), isoschizomeric restriction endonuclease

INTRODUCTION

The comet assay has a long history of being used to assess the effects of various endogenous and exogenous substances on DNA damage and repair. Since, Ostling and Johanson (1984) showed that DNA from ϒ-irradiated cells migrate toward the anode due to the relaxation of the DNA supercoils the comet assay has been modified numerous times. These modifications range from altering the pH of the electrophoresis buffer (Calini et al., 2002), to exposing cells to various chemicals to assess the DNA repair capacity, to treatment of nucleoids with restriction enzymes (Andersson and Hellman, 2005) and even protein extracts to assess the effect of a given substance on DNA repair (Collins et al., 2001; van Dyk et al., 2010). Together with the still widely used standard comet assay, as described by Singh et al. (1988), the variety of modifications made to the comet assay perfectly showcase the adaptability and applicability of this technique.

The comet assay is an affordable and flexible method which can be easily adapted for the measurement of global DNA methylation. DNA methylation is not only important for maintaining genome stability but also plays an important role in gene regulation (Bird, 2002; Nag and Smerdon, 2009). DNA methylation is an epigenetic event which involves the chemical modification of DNA wherein the DNA sequence is not changed. In mammalian cells DNA methylation occurs at the cytosine residue of the CpG dinucleotide pair following each cycle of DNA replication and involves the addition of a methyl group at the carbon-5 position of cytosine through the action of DNA methyltransferases (DNMTs; Turker and Bestor, 1997; Espada and Esteller, 2010; Tost, 2010). DNA methylation patterns can be established on a global or gene-specific level in accordance with regulatory needs (Bird, 2002). The majority of CpGs in the genome are methylated, with the exception of CpG-islands which tend to remain hypomethylated in adult cells except on the inactivated X chromosome (French et al., 2009; Espada and Esteller, 2010). These CpG islands are characterized by relatively high CpG density. If the epigenetic processes are not correctly regulated, it may lead to changes in DNA methylation and histone modification patterns that disrupt important cellular processes, including gene expression, DNA repair and tumor suppression (Walsh and Xu, 2006; Li et al., 2007; Brooks et al., 2010; Tost, 2010).

The adaption of the comet assay to measure global methylation relies on the isoschizomeric properties of the two restriction enzymes: MspI and HpaII. These two isoschizomeric restriction enzymes recognize the same tetranucleotide sequence (5′-CCGG-3′) but display differential sensitivity to DNA methylation. HpaII is inactive when any of the two cytosines is methylated, but it digests the hemimethylated 5′-CCGG-3′ at a lower rate compared with the unmethylated sequences. On the other hand, MspI digests 5′-CmCGG -3′but not 5′-mCCGG-3′. These enzyme properties have been employed in other established techniques, such as the cytosine extension assay (CEA) and the luminometric assay (LUMA) for the measurement of global DNA methylation (Pogribny et al., 1999; Karimi et al., 2006). This difference is exploited to assess the global DNA methylation.

Some of the challenges and limitations of the methylation sensitive comet assay as previously reported (Wentzel and Pretorius, 2012) include, limited sample throughput, insufficient enzyme digestion of nucleoids and drying of agarose before enzyme digestion is complete. These challenges and limitations are; however, not unique to the methylation sensitive comet assay but are encountered in other adaptions of the comet assay as well. To address some of these limitations of the comet assay, a medium and high-throughput comet assay was developed (Stang and Witte, 2009; Azqueta et al., 2013; Gutzkow et al., 2013). Here we now describe combining a medium-throughput comet assay and a

low-throughput methylation sensitive comet assay, to produce a methylation sensitive medium-throughput comet assay. This can then be used to assess the global DNA methylation status of single cells.

MATERIALS AND METHODS

CULTURE CONDITIONS AND METABOLITE TREATMENT

HepG2 cells were cultured in Dulbecco's modified essential medium (D-MEM; Hyclone) containing 10% foetal bovine serum (FBS; Lonza), 1% penicillin/streptomycin (Lonza), 1% 200 mM L-Glutamine (Lonza) and 1% non-essential amino acids (Lonza). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. For metabolite treatment, cells were seeded in 1.9 cm^2 wells (24 well plate; Nunc^{TM}) and cultured until confluent. The cells were subsequently cultured in the presence of 0.01 mM 5-azacytidine (5-Aza-dcR; Sigma–Aldrich) for 24 h. Following treatment, cells were harvested using 1× trypsin (Lonza).

CYTOSINE EXTENSION ASSAY

The CEA was performed according to the method described by (Wentzel et al., 2010). Genomic DNA was isolated from 5-Aza-dCR treated cells using the DNeasy (blood and tissue) kit (Qiagen). The isolated DNA was subsequently separately digested with the endonucleases MspI and HpaII (Fermentas). The restriction enzyme mixture consisted of 1 μl of 1× Tango buffer (per 5 U of enzyme), 500 ng/μl DNA, and 10 U of enzyme (MspI/HpaII) in a final volume of 20 μl. The enzyme reaction was performed at 37°C for 1 h followed by heat inactivation (65°C for 15 min). The CEA reaction mixture consisted of 5× Taq buffer, 25 mM $MgCl_2$, 5 U of GoTaq enzyme (Promega), and 0.1 μl of [^{3}H] deoxycytidine triphosphate (dCTP; GE Healthcare) in a final volume of 15 μl. Subsequently 5 μl of the digested DNA was added to the 15 μl of the CEA reaction mixture and incubated for 1 h at 56°C for the cytosine incorporation. The samples were transferred to Whatman DE-81 ion exchange filters (Whatman) and washed three times with 1× phosphate-buffered saline (PBS). The filters were air-dried at room temperature overnight. Scintillation counting in 9 ml Ultima Gold^{TM} XR (Perkin Elmer®) was performed in a liquid scintillation analyzer (Perkin Elmer® and Quantasmart^{TM} versio 3.00.5 Tri-Carb^{TM} LSC software). Background counts were subtracted from enzyme-treated samples, and the results were expressed as relative [^{3}H] dCTP incorporation/0.5 mg of DNA and presented as percentage change from control samples. All samples were counted twice, and the average was calculated with sigma = 2%. The values were expressed as disintegrations per minute (dpm). Experiments were performed in triplicate.

LOW-THROUGHPUT METHYLATION SENSITIVE COMET ASSAY

Modifications were made to the alkaline comet assay to detect changes in the levels of DNA methylation in single cells (Wentzel et al., 2010), using the 1 gel/slide format. During the harvesting process, cells are exposed to trypsin which may negatively influence the integrity of cells. Harvested cells were incubated in the D-MEM (containing 10% FBS) for 1 h at 37°C in an orbital shaker to recuperate from the trypsin harvesting process. An 50 μl aliquot of the cell sample was mixed with 100 μl (15–20 cells/μl) of 0.5% low-melting-point agarose (LMPA; Fermentas) followed by the application of 100 μl of this solution to a frosted glass slide that had been pre-coated with a thin layer of 1% high-melting-point agarose (HMPA; Sigma–Aldrich). The slides were left at room temperature for the LMPA to set. The slides were subsequently submerged in lysing solution (consisting of 5M sodium chloride (NaCl; Sigma–Aldrich), ethylenediaminetetraacetic acid (EDTA; Sigma–Aldrich), 10% dimethyl sulfoxide [DMSO; Merck) and 1% Triton X-100 (Merck)] at 4°C for 16 h to prepare nucleoids. The methylation sensitive comet assay employs the isoschizomeric restriction enzymes HpaII and MspI (Fermentas). To ensure favorable conditions for enzyme digestion, the slides were soaked in restriction enzyme reaction buffer (10 mmol/L Tris–HCl (Sigma–Aldrich), 10 mmol/L NaCl, 1 mmol/L mercaptoethanol (Sigma–Aldrich), and 2 mmol/L EDTA) for 10 min. Each enzyme mixture was composed of 1.5 unit of MspI or HpaII, 10 μl of Tango buffer (Fermentas) and filled to 100 μl with molecular grade H_2O. 100 μl of this enzyme mix was subsequently applied to each slide and covered with a glass cover slip. The slides are then placed in a damp plastic container lined with towel paper that was preheated to 37°C. After 5 min of incubation the slides are covered with towel paper soaked in reaction buffer to keep the slides from drying out while incubating for another 20 min. After incubation and removal of the coverslips, the slides were put into the electrophoresis tank and covered with electrophoresis buffer (5 mol/L NaOH and 0.4 mol/L EDTA). Electrophoresis took place at 30 V and 300 mA (between 0.8 and 0.9 V/cm) for 45 min at 4°C, after which a pH neutralization step was performed by soaking the slides in 0.4 M Tris–HCl buffer (pH 7.5) for 15 min. Finally, the nucleoids were stained with ethidium bromide (10 μg/ml) for 1 h at 4°C and rinsed with distilled water. The comet images were captured with an Olympus IX70 fluorescence microscope (200× magnification) and scored using Comet IV computer software version 4.3.1 (Perceptive Instruments Ltd). At least 200 comets were randomly scored per slide and the percentage of DNA migrating from the comet head (tail intensity) was measured for each comet scored. Experiments were performed in triplicate with two independent repeats.

MEDIUM-THROUGHPUT METHYLATION SENSITIVE COMET ASSAY

For the medium-throughput methylation sensitive comet assay, a 12-well gasket (Severin Biotech) was used for the preparation of the comet slides and perform enzyme digestion. For cellular repair, the harvested HepG2 cells were incubated in D-MEM nutrient medium (containing 10% FBS) at 37°C in an orbital shaker for 1 h. Frosted glass sides were pre-coated with 300 μl, 1% high melting point agarose (HMPA) and left to dry at room temperature for at least 1 h. The precoated slide was then placed into the 12-well gasket. Following the repair phase, a 50 μl aliquot of the cell sample was mixed with 100 μl of 0.5% low melting point agarose (LMPA) maintained at 40°C. A volume of 20 μl (∼15–20 cells/μl) of this mixture was cautiously applied to each well and the aluminium gasket was placed on ice for 5 min for the LMPA to set. The nucleoids were exposed by adding 150 μl of lysis solution directly to each well and incubated at 4°C for 1 h. Following cell lysis, each well was washed with 1x PBS (Sigma–Aldrich)

at least twice. Nucleoids were treated with *Fast Digest* versions of the restriction enzymes HpaII and MspI (Fermentas). Each enzyme mixture was composed of 5 μl of MspI or HpaII, 5 μl of FB enzyme buffer (Fermentas) and filled to 50 μl with molecular grade H_2O. Then 50 μl of this enzyme mixture was applied to each well and sealed with the silicone cap. The 12-well gasket was incubated at 37°C for 30 min. Alternatively, a 1.0–1.5 mM solution of proteinase K (Qiagen) can also be employed to unwind the nucleus prior to enzyme digestion. This step contributes to making restriction enzyme recognition sites more accessible for MspI and HpaII. After incubation, the frosted glass plate was removed from the gasket and placed in electrophoresis buffer at 4°C. After 30 min, electrophoresis was performed at 30 V and 300 mA (between 0.8 and 0.9 V/cm) for 45 min at 4°C. Electrophoresis was followed by a pH neutralization step by soaking the slides in 0.4 M TrisHCl buffer (pH 7.5) for 15 min. Finally the nucleoids were stained with ethidium bromide (10 μg/ml) for one hour at 4°C and thoroughly rinsed with distilled water. The comet images were captured with an Olympus IX70 fluorescence microscope (200× magnification) and scored using the Comet IV computer software version 4.3.1 (Perceptive Instruments Ltd). At least 400 comets were randomly scored per sample (between 50 and 100 comets per well) and the percentage of DNA migrating from the comet head (tail intensity) was measured for each comet scored. No less than nine replicates of three independent experiments were performed for each sample.

STATISTICAL ANALYSIS

Statistical analysis was done with Prism 5 (GraphPad). For the Medium-throughput methylation sensitive comet assay, at least nine replicates were performed per sample and a minimum of 400 comets per sample were used for statistical analysis. Outliers were removed using the modified Thompson Tau method (Cimbala, 2011). In order to determine the distribution properties of the percentage CpG methylation, the bootstrap method was employed. A bootstrap replication number of 10,000 were employed with a 95% confidence interval. Percentage CpG methylation was calculated using the ratio between the average percentage tail DNA of HpaII- and MspI-digested DNA, that is, [(100–HpaII\MspI × 100) – control], where HpaII and MspI are the average percentage tail DNA of HpaII- and MspI-digested nucleoids, respectively.

RESULTS

The methylation sensitive comet assay is based on the difference in sensitivity to DNA methylation of the two isoschizomeric restriction endonucleases HpaII and MspI. In theory, when these restriction enzymes are used in the comet assay, a higher level of methylation of the CpG dinucleotides should result in a larger difference in the amount of DNA in the comet tails of HpaII-digested nucleoids versus MspI-digested nucleoids. From **Figure 1** it is evident that the treatment of agarose-embedded nucleoids with MspI indeed resulted in markedly more comet tail DNA relative to the undigested control. Similarly, a smaller but still significant, increase in the tail DNA is observed following HpaII treatment.

To improve the low-throughput methylation sensitive comet assay, a 12-well gasket was used for the preparation of the comet slides and enzyme digestion. The original low-throughput and

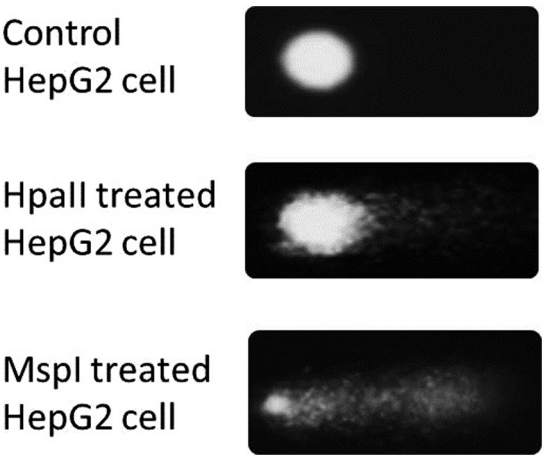

FIGURE 1 | Comets created by the treating nucleoids with the isoschizomeric enzymes MspI and HpaII.

modified medium-throughput comet assays were then compared. The results are expressed as percentage CpG methylation and are calculated using the ratio between the average percentage tail DNA of HpaII- and MspI-digested DNA. The results of the two methylation sensitive comet assays were validated using the CEA on DNA isolated from the remaining cells of the same batch used for the comet assay (**Figure 2**). The calculated percentage CpG methylation is 62.2 and 58.6% for untreated cells and 44.0 and 34.6% for 5-Aza-dcR-treated cells detected by the low-throughput and medium-throughput methylation sensitive comet assays, respectively. For the CEA data set, the percentage CpG methylation is 60.2% for untreated cells and 34.0% for 5-Aza-dcR-treated cells. A comparison of the distribution of the percentage CpG methylation of the low-throughput methylation sensitive comet assay in comparison to the medium-througput methylation sensitive comet assay is depicted in **Figure 3**. The area between the first- and third quartile for percentage CpG methylation is smaller in data generated with the medium-throughput methylation sensitive comet assay in contrast to the low-throughput method, in which percentage CpG methylation is more widely distributed.

DISCUSSION

Although a variety of techniques are used to measure global DNA methylation patterns, most of these techniques are expensive and platform specific (Shen and Waterland, 2007; Lisanti et al., 2013). The comet assay is a cost-effective, sensitive, and simple technique, which is traditionally used for analyzing and quantifying DNA damage in individual cells (Fairbairn et al., 1995; Azqueta et al., 2011). Nowadays this method is regularly used in biomonitoring and mechanistic studies in a large range of *in vitro* and *in vivo* systems (Dusinska and Collins, 2008; Valverde and Rojas, 2009; Cemeli and Anderson, 2011). The comet assay is also widely used for genotoxicity studies and determining DNA repair capacity and a variety of DNA lesions can be detected, including DNA double strand breaks (DSB) and single strand breaks (SSB), as well as alkali-labile sites (Fairbairn et al., 1995; Collins and Gaivao, 2007).

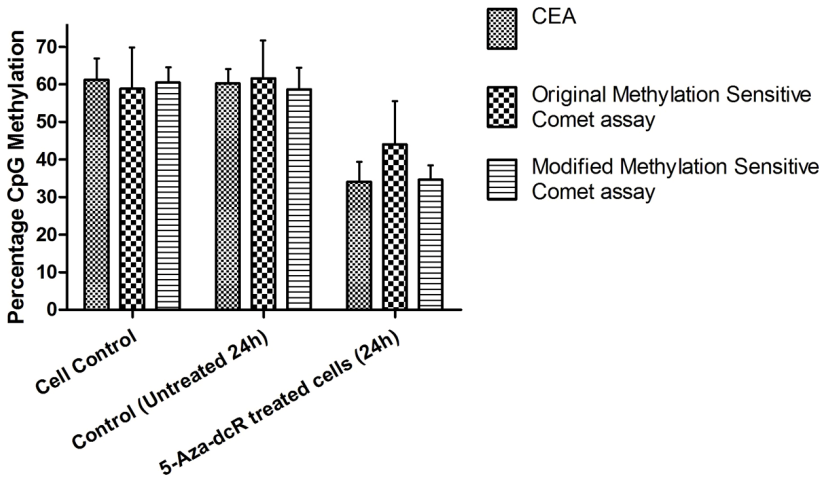

FIGURE 2 | DNA methylation of HepG2 cells treated with 5-Aza-dcR. Comparison between global CpG methylation of cells in culture under normal conditions and treated with the demethylation agent 5-Aza-dcR using the two methylation sensitive comet assays and the CEA. All experiments were at least performed in triplicate with two independent repeats.

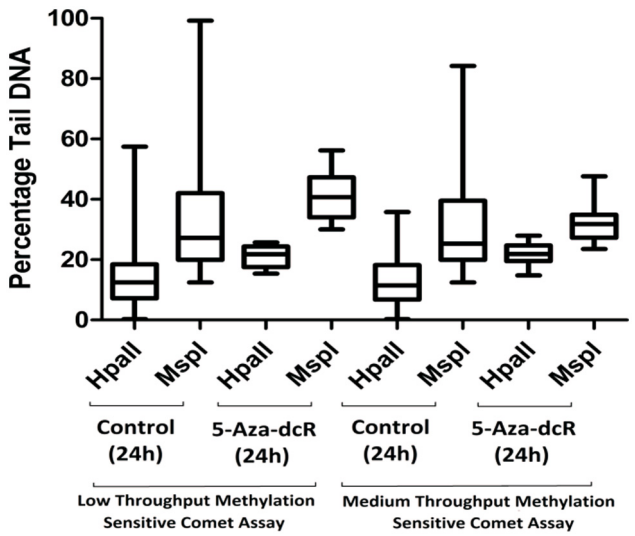

FIGURE 3 | A comparison of the percentage tail DNA in comets of cells cultured under normal conditions in comparison with cells treated with the demethylation agent 5-Aza-dcR as measured with the low- and medium-throughput methylation sensitive comet assays. A bootstrap replication number of 10,000 were employed with a 95% confidence interval. All experiments were at least performed in triplicate with two independent repeats.

The use of specific restriction endonucleases with the comet assay expands the flexibility of the method. The comet assay can be modified through the use of lesion specific restriction endonucleases to detect specific base modifications as DNA SSB (Epe et al., 1993; Tice et al., 2000; Collins and Gaivao, 2007; Collins, 2009; Speit et al., 2009). In a similar way the comet assay can be modified to measure DNA methylation by using methylation sensitive restriction endonucleases. By doing this it is possible to simultaneously measure global as well as CpG island DNA methylation and DNA damage and repair in a variety of cells (Wentzel et al., 2010).

The use of methylation sensitive restriction endonucleases can modify the traditional alkaline comet assay to be methylation sensitive. Similar to the CEA that measures global DNA methylation (Pogribny et al., 1999), the methylation sensitive comet assay also employs the isoschizomeric restriction endonucleases HpaII and MspI. As previously mentioned, these enzymes recognize the same tetranucleotide sequence (5′-CCGG 3′) but display differential sensitivity to DNA methylation (**Figure 1**). Unmethylated DNA is digested by HpaII, however, when either of the two cytosines are methylated HpaII will not digest the DNA. When the DNA is hemimethylated, i.e., only one of the two complimentary strands are methylated, HpaII will digest the DNA, but at a slower rate than digestion of unmethylated DNA. Conversely, MspI will digest methylated DNA, but only 5′-CmCGG-3′ and not 5′-mCCGG-3′ (Tost and Gut, 2010).

Even though theoretically the percentage tail DNA following MspI treatment represents all of the 5′-CCGG-3′ sites in the DNA, it is important to note that when using MspI and HpaII only the methylated cytosines outside of CpG islands are quantified as these enzymes tend to mainly recognize sequences outside of CpG islands. Cytosines within these regions tend to be methylated whereas cytosines in the CpG islands tend to be unmethylated (Shen and Waterland, 2007). The global 5′-CCGG-3′ methylation can be calculated by the HpaII/MspI ratio. Compensation is made for DNA damage prior to enzyme treatment by subtracting the percentage tail DNA from the control samples.

In the current study the previously modified method (Wentzel et al., 2010) was further adapted by using *Fast Digest* versions of the HpaII and MspI restriction endonucleases and the 12 gels/slide format of the comet assay (Shaposhnikov et al., 2010). In short, HepG2 cells were exposed to the demethylating agent 5-Aza-dcR

for 24 h as exposure to this demethylating agent causes a decrease in the percentage global DNA methylations. Results from the conventional 1 gel/slide format (low-throughput comet assay) was compared to the use of 12 gels/slide format (medium-throughput comet assay) using the *Fast Digest* versions of HpaII and MspI and validated with the established CEA (Pogribny et al., 1999).

A similar decrease in the percentage CpG methylation following 5-Aza-dcR treatment for the CEA and the medium-throughput methylation sensitive comet assay (26.2 and 24 %) and comparatively lower decrease in percentage CpG methylation for the low-throughput methylation sensitive comet assay following 5-Aza-dcR treatment was seen (**Figure 2**). **Figure 3** furthermore showed that the distribution of the data was also better for the medium-through put comet assay compared to the low-throughput comet assay.

The results for the medium-throughput methylation sensitive comet assay and the CEA following 5-Aza-dcR treatment are similar due to the fact that the enzyme digestion conditions are nearer to that recommended by the manufacturer and closer to the conditions used in the CEA. The enzyme digestions are performed in individual wells and a silicon cover is placed over the gasket forming a lid over each individual well during incubation. In the low-throughput method the enzyme/buffer mixture is spread over the entire gel, a glass cover slide is placed over the frosted glass slide and it is then incubated in a damp plastic container. In the later method the enzyme/buffer mixture tends to evaporate, which changes the enzymatic reaction conditions.

The use of the 12-gels/slide instead of the traditional 1 gel/slide not only upgraded the comet assay to a medium-throughput method, the 12-well gasket greatly improved the restriction enzyme digestion conditions and considerably reduced consumable use. The deployment the *Fast Digest* versions of the restriction enzymes HpaII and MspI, further also improved nucleoid digestion and reduced incubation time. This modified method also overcomes "edge-effects" as observed when the traditional frosted glass slides are used.

CONCLUSION

The difference in methylation sensitivity of the isoschizomeric restriction endonucleases HpaII and MspI may be exploited to demonstrate the feasibility of using the comet assay to measure global DNA methylation level in individual cells. In the present study we showed that the comet assay can be modified to measure global DNA methylation in single cells in a medium-throughput manner. The use of the 12-well gasket to perform the enzyme digestions offers more ideal conditions for enzyme digestion and overcomes some of the limitations that are faced when restriction enzymes are used in conjunction with the comet assay, such as "edge-effects," sub-optimal enzyme reaction conditions and gel drying.

The use of the comet assay over other methods such as CEA for the measurement of global DNA methylation offers the advantage that it is less expensive. Furthermore, DNA methylation is tissue specific (Pogribny et al., 1999) and this method can be used to measure the changes in the global DNA methylation pattern of a variety of cells under different physiological conditions on a single cell level.

The versatility of the comet assay is further expanded through the modifications made in this study, increasing the number of observations that can be made with a single experiment and reducing the amount of labor and inter-experimental variability.

AUTHOR CONTRIBUTIONS

Angélique Lewies, Etresia Van Dyk, Johannes F. Wentzel, and Pieter J. Pretorius (principal investigator) designed the study. Angélique Lewies, Etresia Van Dyk, Johannes F. Wentzel performed the experiments, processed the data, and wrote the manuscript.

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FISH comets show that the salvage enzyme TK1 contributes to gene-specific DNA repair

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Thymidine kinase 1 (TK1) is a salvage enzyme that phosphorylates thymidine, imported from surrounding fluids, to create dTMP, which is further phosphorylated to the DNA precursor dTTP. TK1 deficiency has for a long time been known to cause increased cellular sensitivity to DNA damage. We have examined preferential strand break repair of DNA domains in $TK1^+$ and $TK1^-$ clones of the Raji cell line, by the Comet-FISH technique, in bulk DNA and in the actively transcribed tumor suppressor (TP53) and human telomerase reverse transcriptase (hTERT) gene regions, over 1 h after 5Gy γ-irradiation. Results showed that repair of the TP53 and hTERT gene regions was more efficient in $TK1^+$ compared to $TK1^-$ cells, a trend also reflected to a lesser degree in genomic DNA repair between the cell-lines. The targeted gene-specific repair in TK^+ cells occurred rapidly, mainly over the first 15 min repair-period. Therefore, TK1 is needed for preferential repair of actively transcribed regions, through a previously unsuspected mechanism. In principle, TK1 could exert its protective effects through supply of a supplementary dTTP pool for accurate repair of damaged genes; but Raji $TK1^+$ cells in thymidine free media still show preferential repair of transcribed regions. TK1 therefore does not exert its protective effects through dTTP pools, but through another unidentified mechanism, which affects sensitivity to and mutagenicity by DNA damaging agents.

Keywords: FISH comet, gene-specific repair, radiation damage, thymidine kinase

INTRODUCTION

Human thymidine kinase 1 (TK1) is a salvage enzyme that phosphorylates thymidine to create dTMP, which is later converted by thymidylate kinase and nucleoside diphosphate kinase to dTTP, a precursor for DNA metabolism (Segura-Pena et al., 2007). This is usually the minor pathway for dTTP synthesis, subsidiary to the *de novo* pathway in which ribonucleotide reductase converts UDP to dUDP, which is dephosphorylated to dUMP and then methylated by thymidylate synthase to dTMP. TK1 is not essential for viability (Dobrovolsky et al., 2003). A related enzyme, TK2, is mitochondrial and irrelevant to nuclear events.

While the functions of TK1 are clearly related to the processes of DNA replication and cell proliferation, many studies in the literature demonstrate a protective role for the protein during cellular responses to DNA damage. Human and rodent *in vitro* studies show that deficiency of the protein causes increased sensitivity to a diverse range of DNA damaging agents, including ionizing radiation (McKenna and Hickey, 1981; McKenna and Yasseen, 1982; McKenna et al., 1988; al-Nabulsi et al., 1994; Best et al., 1994; Wakazono et al., 1996). In this context, it is noticeable that TK1 mRNA is induced not only in S phase and G2 but also following ionizing radiation, causing a concomitant increase of enzymatic activity (Boothman et al., 1994; Wei et al., 1999; Castro Kreder et al., 2002; Haveman et al., 2006). More recently, it has been shown that TK1 is upregulated in different tumor types in response to DNA damage, and that the cellular response to genotoxins causes nuclear localization of TK1; an interesting finding given that the salvage enzyme has previously been regarded as solely cytoplasmic (Chen et al., 2010). These studies suggest that TK1 may somehow affect DNA metabolism in a way not obviously explained by its salvage role.

The protective effect of TK1 might be due to its maintaining of the efficiency of DNA repair during recovery from genotoxic insults. TK1 deficiency not only restricts the dTTP pool but upsets the balance of all four dNTP precursors (Wilkinson and McKenna, 1989). Deficiency of the TK1 regulated dTTP pool causes decreased viability and increased mutation after treatment with mutagenic agents (Wakazono et al., 1996; Kubota et al., 1998; Hyland et al., 2000). Analagously, a reduction in dTTP pools by silencing of thymidylate kinase also sensitizes cells to DNA damage (Hu and Chang, 2008). Recent work in colon carcinoma cells (Chen et al., 2010) showed that knockdown of TK1 decreases the efficiency of double-strand DNA break repair during recovery from DNA damage. Chen et al. (2010) also found that the TP53 status of the tumor cells affected the level of TK1 after DNA damage.

We address in this study TK1's effect on the kinetics of repair after DNA damage, in particular the repair of DNA damage occurring in specific areas of the genome. Many studies have documented that repair occurs at a more accelerated rate in transcribed

gene regions such as TP53, compared to that of total DNA (reviewed by Sarasin and Stary, 2007). Such transcription-coupled repair has the capacity to reduce mutations in vital domains of the genome. While earlier DNA repair studies using murine cells have shown that TK1 deficiency does not prevent bulk excision repair from occurring after genotoxic insult (McKenna and McKelvey, 1986), there has been no evidence for its effect on the faster repair that occurs with damage to specific gene regions. The Comet assay is an ideal method for investigations of the kinetics of repair of damage induced in nuclear DNA by ionizing radiation. The strand breaks induced are typically rejoined quickly, most breaks disappearing within 30 min (reviewed by Frankenberg-Schwager, 1989). The method when combined with the use of fluorescent hybridization probes (Comet-FISH) allows the study of repair kinetics in gene-regions of interest by quantifying the rate of strand break repair within such target genes (Santos et al., 1997; McKelvey-Martin et al., 1998; McKenna et al., 2003; Horvathova et al., 2004; Glei et al., 2007).

This investigation was carried out to determine whether TK1 may affect the damage response in two selected transcribed gene regions [TP53 and human telomerase reverse transcriptase (hTERT)] both of which are actively transcribed by Raji $TK1^+$ and $TK1^-$ clones, and thereby to provide further insight into potential mechanisms of mutagenesis and carcinogenesis induced by misrepair of DNA. Human TP53 is a well-characterized tumor suppressor gene and is located on the short arm of chromosome 17 (17p13.1; Matlashewski et al., 1984). TP53 is induced by γ-irradiation (McKay et al., 1999) and is in a domain seen to be rapidly repaired after γ-irradiation in comparison to other genes (McKenna et al., 2003, 2012). The hTERT gene (5q15.33), which codes for hTERT, the catalytic subunit of the telomerase enzyme, is upregulated in the majority of cancer cells (Hiyama and Hiyama, 2003); strand break repair of γ-irradiation damage to the hTERT gene domain is likewise rapid (McKenna et al., 2012).

The position of FISH signals within the Comet head or tail indicates whether or not damage has occurred, or not yet been repaired, to the gene-region selected by the probe; this can be compared to damage in global DNA. In this study we applied the Comet-FISH protocol to study DNA repair in $TK1^+$ and $TK1^-$ clones of the human lymphoblastoid cell line Raji. The possibility that TK1 exerts its protective effects through the dTTP pool was also investigated by growing Raji $TK1^+$ cells in thymidine free media. Repair was followed over a 1-h period following exposure to 5Gy γ-irradiation.

MATERIALS AND METHODS

CELL-LINES AND CELL CULTURE

$TK1^-$ and $TK1^+$ clones of the Raji lymphoblastoid cell line were grown in RPMI 1640 culture media, supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). Raji cell-lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). In thymidine free experiments, $TK1^+$ cells were first washed in PBS, then cultured in thymidine free media containing dialyzed FBS to remove the salvage dTTP pool. The Werner syndrome (WS) cell line was obtained from the Coriell Cell Repository (Camden, NJ, USA) and maintained in minimum essential medium, supplemented with 15% FBS and glutamine, penicillin, and streptomycin as above. The GM38 normal human fibroblast cell-line was obtained from the Human Genetic Msitory (Camden, NJ, USA) and cultured in Eagle's minimum essential medium supplemented with 20% FBS, 4% essential amino acids, 2% non-essential and glutamine, penicillin, and streptomycin as above. The GM38 cell line actively expresses TP53 (Glei et al., 2007) and served both as a positive and negative PCR control to study TP53 and hTERT gene expression, respectively. The WS cell line is immortalized with the hTERT gene and therefore served as a PCR positive control to examine hTERT gene expression in Raji cells.

CHARACTERIZATION OF RAJI CELLS

Raji $TK1^-$ cells were cultured in 5 μg/ml trifluorothymidine (TFT; Sigma, Poole, UK), which is lethal to $TK1^+$ cells, to confirm the cellular phenotype. TK assays were used to determine the activity of TK1 in Raji cells (McKenna et al., 1985). Metaphase spreads ($n = 100$) were examined to quantify chromosome numbers in Raji cells for general characterization and to correlate with later FISH spot numbers (McKenna et al., 2003).

hTERT AND TP53 GENE EXPRESSION

Total RNA was extracted from each of the four cell lines using an RNeasy Mini kit (Qiagen, Mississauga, MD, USA); integrity was verified by 1% gel electrophoresis and quantity and quality determined using a NanoDrop ND-1000 UV-VIS Spectrophotometer (Wilmington, DE, USA). Complementary DNA was generated using a Superscript II RNase H-Reverse Transcriptase Kit (Invitrogen, Renfrew, UK) according to the manufacturer's protocol. β-actin served as the internal control in RTPCR reactions. Template cDNA was amplified for PCR using GoTaq DNA Polymerase (Promega, Southampthon, UK) in the presence of primers specific for the β-actin gene: 5′AGAAAATCTGGCACCACACC-3′ (sense) and 5′CCATCTCTTGCTCGAAGTCC-3′ (anti-sense), or primers specific for the hTERT gene: 5′CTCACCTTCAACCGCGG-3′ (sense) and 5′TTGCTGAAATGGGAGCT-3′ (anti-sense). Reaction conditions for β-actin were 40 cycles of denaturation at 96°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 10 min; conditions for hTERT were 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s. Platinum TAQ DNA polymerase (Invitrogen, Renfrew, UK) was used to amplify the TP53 gene using the following primers: 5′-TCACTGCCATGGAGGAG-3′ (sense) and 5′-TCAGTGGGGAACAAGAAG-3′ (anti-sense). PCR reactions conditions were: 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 2 min. A final elongation step of 10 min was used to ensure that any remaining single stranded DNA was completely copied.

COMET-FISH ASSAY

For the alkaline Comet assay, cells were embedded in agarose onto Dakin fully frosted microscopic slides (Labcraft, London, UK), and subjected to a dose of 5Gy irradiation using a Cs^{137} source. Following irradiation slides were quickly immersed in repair medium

at 37°C for either 15, 30, or 60 min, then drained and placed in alkaline lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, and 1% Triton X-100 added before use) for 1 h at 4°C. DNA was left to unwind in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) for 20 min. Electrophoresis was conducted for 20 min at 25 V and 300 mA, after which slides were washed with three changes of neutralization buffer (0.4 M Tris, pH 7.5).

Target DNA was prepared for FISH by a 5 min wash with 2X SSC (3 M sodium citrate, pH 5.3), and subsequent dehydration with increasing concentrations of ethanol (70, 85, 100%). Multicolor Comet-FISH was performed using a fragmented locus-specific identifier (LSI) Spectrum-Orange-labeled TP53 DNA probe spanning a 140 kb region containing the 20 kb TP53 gene (Vysis, Surrey, UK), in conjunction with a LSI direct labeled Spectrum Green hTERT probe, spanning a 180 kb region containing the 40 kb hTERT gene (Q-Biogene, Cambridge, UK). Equal concentrations of a TP53 and hTERT probe mix were applied to each slide. Co-denaturation of DNA was performed at 80°C (2 min) followed by a 16-h hybridization period at 37°C in a dark, humidified chamber. Following hybridization, excess probe was removed at 45°C using three 10 min washes of 50% formamide and 2X SSC, a 10 m wash with 2X SSC and a final 5 min 2X SSC and 0.1% Igepal wash. Slides were left to air dry for 1 h then counterstained with 16 μl 4′6-diamidino-2-2phenylindole (DAPI) for immediate observation. All reagents for the Comet-FISH assay were purchased from Sigma, Poole, UK unless otherwise stated.

COMET FISH MICROSCOPY AND ANALYSIS

The slides were viewed using an epifluorescence microscope (Nikon Eclipse E400) using a triple bandpass filter (Chroma HiQ) that enabled the simultaneous detection of DAPI (overall genome) and both spectrum orange (TP53 gene region) and spectrum green (hTERT gene region) for the enumeration of FISH signals. Standard Comet parameter measurements, including % comet tail DNA, were recorded using the Komet 5.0 digital imaging system (Kinetic Imaging Ltd., Liverpool, UK).

Studies of repair of bulk DNA selected the measurement % comet tail DNA, as the amount of DNA in the tail (relative to the comet head) is proportional to the number of γ-irradiation induced strand breaks. For DNA damage and repair to the p53 and hTERT gene regions the numbers of fluorescent probes detected in the comet head and tail were quantified over timed intervals during a 1-h incubation period. The repositioning of the gene-specific signals from the comet tail into the head over the incubation period provides evidence for repair of lesions occurring within and around the TP53 and/or hTERT gene locus. The level of DNA repair in the TP53 and hTERT gene region was examined using the parameter % TP53/hTERT signals in the comet tail.

STATISTICAL ANALYSIS

One slide was analyzed from each dose point, and per slide 50 comets were scored. Three independent experiments were conducted to generate each data point (150 cells scored in total per cell line). The normality distribution of the entire comet-FISH dataset was visually inspected from normal probability plots and evaluated using the Shapiro–Wilk *W*-test. The Mann–Whitney test was applied to evaluate comparisons between the non-parametric comet-FISH dataset for the $TK1^+$ and $TK1^-$ clones. A difference with a $p < 0.05$ was deemed significant. Results were analyzed using GraphPad Prism version 5 (UK).

RESULTS

CHARACTERIZATION OF RAJI CELLS

The Raji cells used are from lines that were originally created in the 1990s: we have therefore checked that they have retained their original phenotypes. The $TK1^+$ line retain sensitivity to the toxic thymidine analog trifluorothymidine (Karran et al., 1990), while $TK1^-$ cells which cannot metabolize it remain resistant. Correspondingly, incorporation of tritiated thymidine is linear in $TK1^+$ cells, negligible in $TK1^-$ (**Figure 1**). The $TK1^-$ nature of the Raji clones, first established several years ago (Hampar et al., 1971), has therefore been maintained.

Metaphase spread analysis revealed that Raji $TK1^+$ and $TK1^-$ cells have a near diploid mainline featuring a population of cells ranging from 42–50 chromosomes. The modal chromosome number for $TK1^-$ was 46 while for $TK1^+$ it was 47. A small, more aneuploid population in both cell-lines increased the overall mean number of chromosomes to 54 and 52 for $TK1^-$ and $TK1^+$ respectively.

We have used TP53 and hTERT as markers for transcribed gene repair: it was therefore necessary to check they are in fact transcribed in both cell lines. They are, as shown in (**Figure 2**). A sample was considered positive for the hTERT gene expression by the presence of a 200 base pair amplicon (**Figure 2C**), as was the case for every cell-line except the fibroblast cell-line GM38. TP53 gene expression was also detected in both Raji clones and the normal fibroblast, GM38, as shown in **Figure 2D** by the presence of a 1220-base pair amplicon. Sequencing data of the TP53 gene in the Raji cell-lines also confirmed that the alleles were identical in both, and therefore any difference in DNA repair could not be accounted for by mutations in this key DNA repair gene (sequencing data not shown, supplementary).

DNA REPAIR IN $TK1^+$ AND $TK1^-$ CELLS

Comet-FISH experiments were evaluated by quantifying the number of TP53 and hTERT hybridization spots located in the comet head or tail of $TK1^+$ and $TK1^-$ at each repair incubation time as shown both in representative images in **Figure 3** and the data in **Table 1**. FISH comet tail spots were almost entirely absent in both un-irradiated controls; and on account of the aneuploidy and the post-replicative elements in the population, the average spot number in the comet head was above two (**Figure 3A**). Post-irradiation, both cell-lines showed an increase in TP53 and hTERT probe signal in the comet tail, which is indicative of damage to both gene regions (**Figures 3B,C**). A notable finding in Raji $TK1^+$ cells (**Table 1**) was a rapid rate of gene-specific repair that featured significant decreases in the number of TP53 and hTERT tail spots at 15 min compared to $TK1^-$ ($p < 0.0001$) and also at 30–60 min ($p < 0.0001$). Repair continued at a slower rate for the remainder of the repair period in TK^+ cells so that by 60 min there was no signal left of either probe in

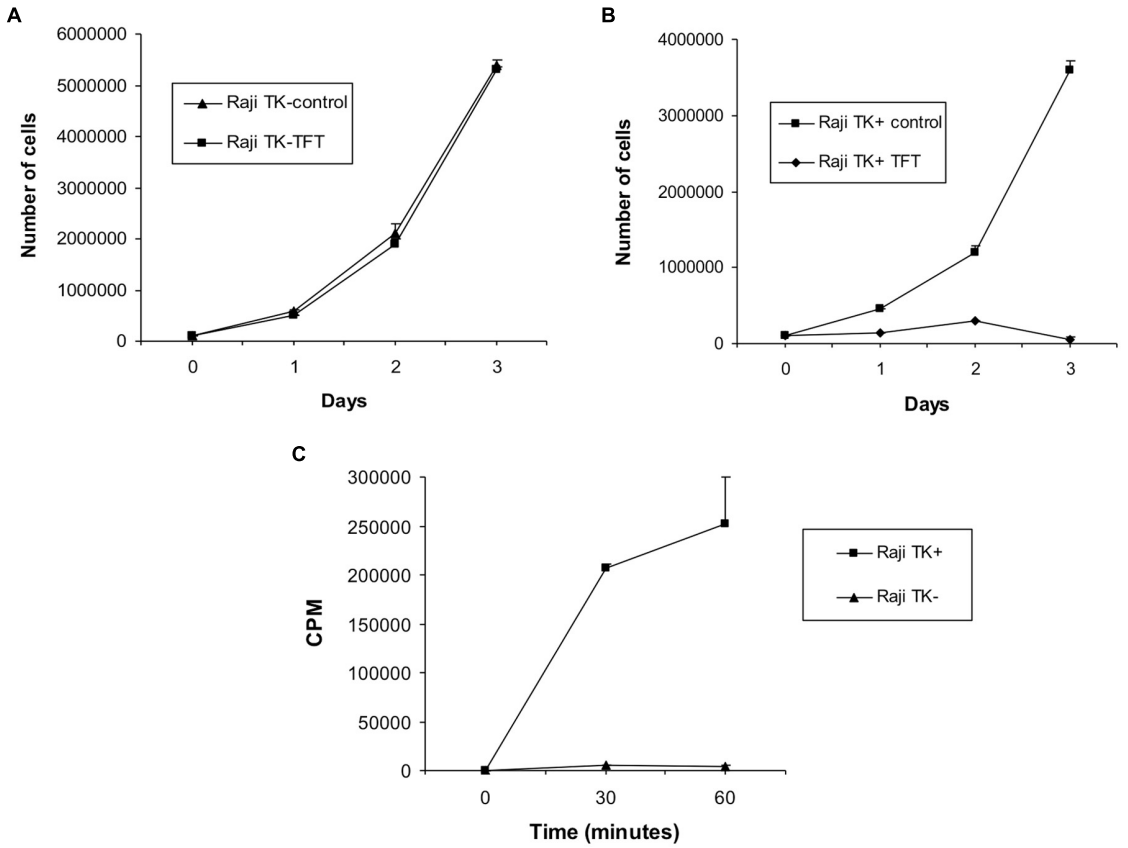


FIGURE 1 | Confirmation of cellular phenotype in Raji cells. (A) Effect of culturing TK1$^-$ cells and **(B)** TK1$^+$ cells in 5 μg/ml TFT compared to untreated control cells. Culture of Raji cells in TFT proved toxic to the TK1$^+$ clones whereas TK1$^-$ cells were resistant. Only cells containing functional TK1 can incorporate TFT and uptake of the chemical results in cell death. Therefore Raji TK1$^-$ cells lack TK1 functional protein. **(C)** Tritium counts obtained from Raji TK1$^+$ and TK1$^-$ clones during the TK assay expressed in counts per minute (CPM). The graph shows that the incorporation of ^{3}H-thymidine is greatly reduced in Raji TK1$^-$ cells compared to TK1$^+$. The ability of cells to incorporate ^{3}H-thymidine into nucleic acids is an approximate measure of cellular thymidine kinase activity; therefore Raji TK1$^-$ cells lack functional TK1 protein. The results in each graph data-point represent the mean ± SEM of three independent experiments.

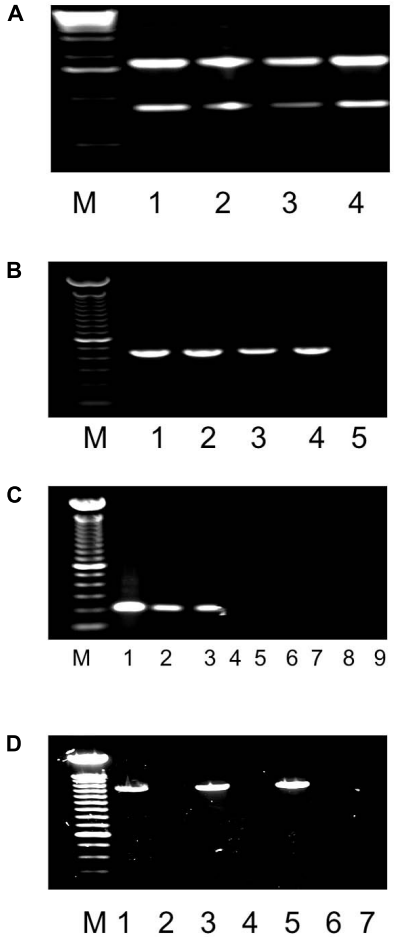
the tail (**Figure 3D**). In comparison, gene-specific repair was found to be stalled in TK1$^-$ cells and the comet-tail FISH signals for each gene probe began to diminish only after 15 min (**Figure 3E**).

The level of strand break repair in the specific gene-regions was compared between TK1$^+$ and TK1$^-$ cells using the parameter "% TP53/hTERT signals in the comet tail" (**Figures 4A,B**). There was found to be a significant reduction ($p < 0.0001$) in the % of TP53 and hTERT tail signals at each time-point in Raji TK1$^+$ cells compared to TK1$^-$ (**Figures 4A,B**). The most prominent occurred at 15 min, with a difference between the Raji clones of 35.18 ± 3.56 for TP53 tail spots and 29.82 ± 4.34 for hTERT tail spots. The delayed reduction of % FISH signals in the comet tail for each gene over the hourly incubation period demonstrates that TK1$^-$ are severely compromised in gene region repair kinetics compared to TK1$^+$.

This may be compared to the levels of radiation-induced strand breaks in bulk genomic DNA, which were reduced over the 1-h repair period in a similar pattern for both Raji TK1$^+$ and TK1$^-$ cells. The percentage of total DNA in the Comet tail ranged from 32 to 8 % in TK$^+$ cells and from 31 to 10.11% in TK$^-$ cells (**Figures 4C,D**). Statistics showed that TK$^+$ cells had significantly but slightly greater levels of bulk DNA repair at both 15 and 30 repair minutes ($p > 0.05$) compared to TK1$^-$ cells.

The level of strand break repair in the specific gene-regions was compared between TK1$^+$ and TK1$^-$ cells using the parameter "% TP53/hTERT signals in the comet tail" (**Figures 4A,B**). There was found to be a significant reduction ($p < 0.0001$) in the % of TP53 and hTERT tail signals at each time-point in Raji TK1$^+$ cells compared to TK1$^-$ (**Figures 4A,B**). The most prominent reduction occurred at 15 min, with a difference between the Raji clones of 35.18 ± 3.56 for TP53 tail spots and 29.82 ± 4.34 for hTERT tail spots. The delayed reduction of % FISH signals in the comet tail for each gene over the hourly incubation period demonstrates that TK1$^-$ are severely compromised in gene region repair kinetics compared to TK1$^+$.

Figure 4C evaluates the mean % comet tail damage in Raji TK1$^+$ cells and shows an accelerated reduction of tail TP53 and



RNA EXTRACTIONS
1. Werner syndrome
2. Raji TK+
3. Raji TK-
4. GM38

B-ACTIN GENE EXPRESSIOI
1. Werner syndrome RTPCR
2. Raji TK+ RTPCR
3. Raji TK- RTPCR
4. GM38 RTPCR
5. PCR water control

HTERT GENE EXPRESSION
1. Werner syndrome RTPCR
2. Raji TK+ RTPCR
3. Raji TK- RTPCR
4. GM38 RTPCR
5-8 RTPCR- controls
9. PCR water control

TP53 GENE EXPRESSION
1. GM38 RTPCR
2. GM38 RTPCR-
3. Raji TK+ RTPCR
4. Raji TK+ RTPCR-
5. Raji TK- RTPCR
6. Raji TK- RTPCR-
7. PCR water control

FIGURE 2 | Investigation of TP53 and hTERT status in Raji cells using RTPCR. (A) RNA extraction. Lane 1 Werner Syndrome (WS), lane 2 TK1$^+$, lane 3 TK1$^-$, lane 4 GM38, M is a 1 kb ladder. **(B)** β-actin gene expression internal control check as detected by a 435 amplicon in all cell-lines. Lane 1 WS, lane 2 TK1$^+$, lane 3 TK1$^-$, lane 4 GM38 and lane 5 PCR water control. M is a 100 bp ladder. **(C)** HTERT gene expression detected by a 200 bp amplicon. HTERT gene expression was found in the WS cell-line, TK1$^+$ and TK1$^-$ cells, but not GM38. Lane 1 WS, lane 2 TK1$^+$, lane 3 Raji TK1$^-$, lane 4 GM38 lane 5 –RTPCR control (-RT) WS, lane 6 –RT TK1$^+$, lane 7 –RT TK1$^-$, lane 8 –RT GM38, lane 9 PCR water control. M is a 100 bp ladder **(D)** TP53 gene expression as detected by a 1220 bp band present in all cells. Lane 1 GM38, lane 2 GM38 –RT, lane 3 Raji TK1$^+$, lane 4 Raji TK1$^+$ –RT, lane 5 Raji TK1$^-$, lane 6 Raji TK1$^-$ –RT, lane 7 PCR water control. M is a 100 bp ladder.

hTERT spots compared to tail bulk DNA. **Figure 4D** demonstrates how reduction of % comet tail DNA damage occurs at more similar rates for both bulk DNA and the TP53 and hTERT gene regions in the TK1$^-$ cell-line. The pattern of repair observed in each gene region would suggest that preferential repair is occurring in the TP53 and hTERT domains of Raji TK1$^+$ but not in TK1$^-$ cells.

EFFECT OF THYMIDINE FREE CULTURE ON PREFERENTIAL REPAIR CAPABILITIES OF RAJI TK1$^+$ CELLS

The growth of TK1$^+$ cells in thymidine free media (Raji TK$^+$ Thy$^-$) had no impact on the proficiency of single strand repair of bulk DNA in Raji TK1$^+$ cells (**Figure 5A**) or gene region-specific repair of TP53 (**Figure 5B**) or hTERT (**Figure 5C**). **Figure 5B** shows TP53 repair in Raji TK1$^+$ Thy$^+$ and Raji TK1$^+$ Thy$^-$ cells. Statistical analysis found no significant difference in the % of TP53 tail signals between TK1$^+$ Thy$^+$ and Raji TK1$^+$ Thy$^-$ cells ($p > 0.05$). Statistical analysis likewise found no significant difference in the % hTERT tail signals at any given time points. These experiments show that culturing of TK1$^+$ cells in thymidine free media has no effect on the level of preferential repair.

DISCUSSION

The misrepair of DNA damage can result in tumorigenesis through mutational activation of proto-oncogenes and inactivation of tumor suppressor genes, causing a variety of human cancer syndromes. Although TK1 deficiency is known to cause a clear increase in DNA damage sensitivity and mutagenicity, and the likelihood of increased carcinogenesis, the DNA repair process on which the salvage enzyme exerts its protective effects remains to be elucidated. While our results in human cells show that TK1 deficiency can reduce the rate of bulk DNA repair, the most prominent finding is the clear role for TK1 in repair of damage occurring in specific gene regions. It is of further interest to monitor DNA repair processes

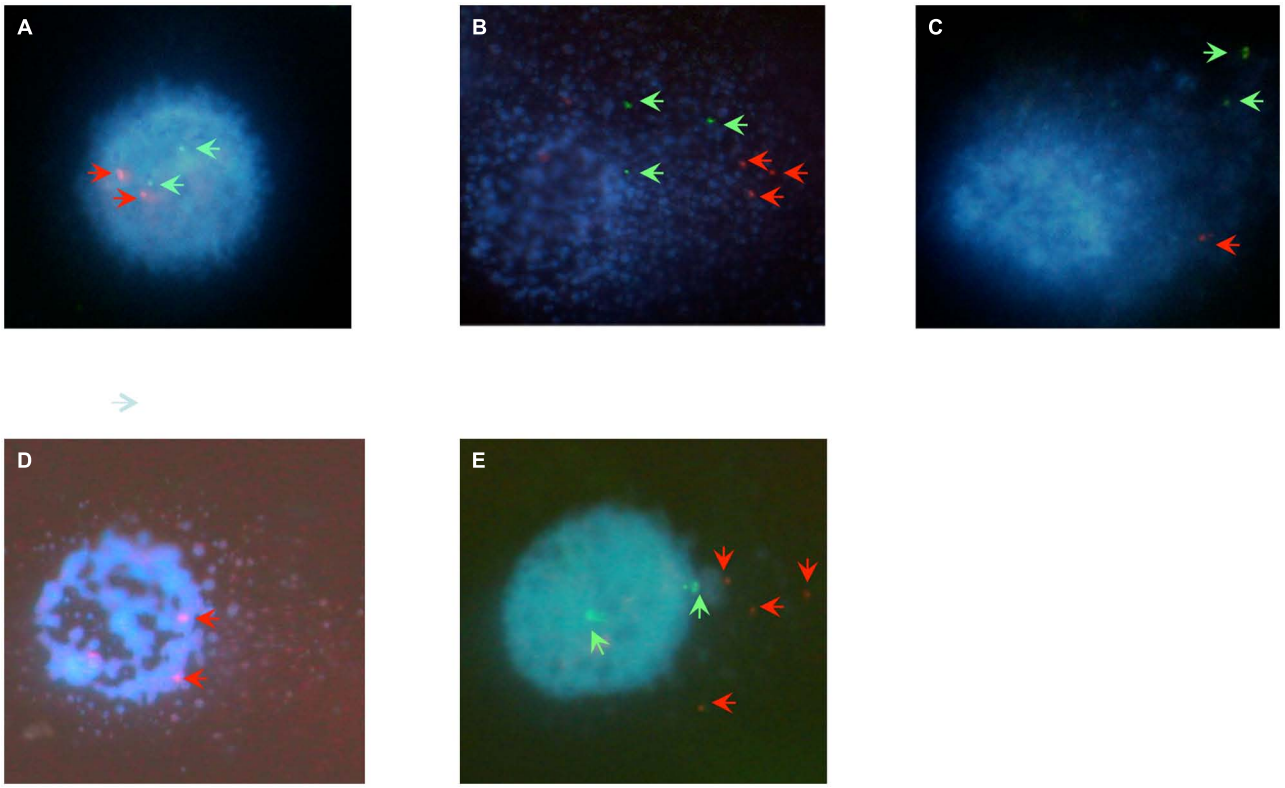


FIGURE 3 | Digital imaging of comet-FISH cells. The position of TP53 and hTERT hybridization spots in the comet head or tail specifies whether it lies in, or close to, a region of intact or damaged DNA. Observations for imaging were made at a final magnification of ×600 (Nikon ×60 Fluor lens). **(A)** An untreated Raji cells displays minimal DNA damage as evidenced by the intact comet head and minimal comet tail. Two TP53 (red arrows) and two hTERT (green arrows) hybridization spots are visible in the intact comet head. **(B,C)** Immediately following 5Gy γ-irradaition a large comet tail is visualized, indicating a large amount of overall DNA damage, with red TP53 and green hTERT hybridization spots in the comet tail indicating that radiation-induced strand breaks have occurred within or close to the vivinity of both genes of **(B)** TK^+ and **(C)** TK^- cells. **(D)**. At 15 min of repair the TK^+ cells have repaired most gene specific damage as depicted here for the TP53 gene region, as evidenced here by the FISH spots being located in the head. **(E)** Raji TK^- cells at 60 min post-irradiation typically feature TP53 and/or HTERT FISH spots located in the comet-head as gene repair has begun to recover. However, FISH tail spots still remain in higher numbers than TK^+, this picture showing an example of damage remaining in the TP53 locci of TK^- cells at 60 min.

in actual cancer cell-lines such as Burkitt's lymphoma-derived Raji, as a full appreciation of the mechanisms that govern DNA repair efficiency in such cells could help lead to the development of novel chemotherapeutic agents, and also help predict patient response to radiotherapy (McKenna et al., 2008).

Table 1 | Data generated by the comet-FISH assay for both Raji TK^+ and TK^-cells at 0, 15, 30, and 60 min following a dose of 5Gy γ-irradiation.

		Repair time-point (minutes)			
Cell-line	**Percentage (%)**	**0**	**15**	**30**	**60**
Raji TK^+	DNA in the comet tail	31.76 ± 1.25	18.36 ± 1.24	12.82 ± 1.41	8.05 ± 0.74
	TP53 comet tail signals	70.05 ± 2.20	22.31 ± 2.61	18.22 ± 3.03	7.53 ± 2.19
	hTERT comet tail signals	77.50 ± 1.89	29.77 ± 3.12	15.03 ± 2.37	6.82 ± 1.88
Raji TK^-	DNA in the comet tail	30.53 ± 0.99	22.98 ± 1.46	15.82 ± 1.46	10.98 ± 0.79
	TP53 comet tail signals	62.28 ± 2.45	57.49 ± 2.41	37.63 ± 3.46	17.12 ± 2.36
	hTERT comet tail signals	74.05 ± 3.16	59.59 ± 3.02	39.87± 3.80	29.82±3.19

Each data point represents the mean value obtained from three independent experiments; 50 cells were quantified per replicate. Comet-FISH experiments were first evaluated by quantifying the number of TP53 and hTERT hybridization spots located in the comet head or tail of $TK1^+$ and $TK1^-$ at each repair incubation time.

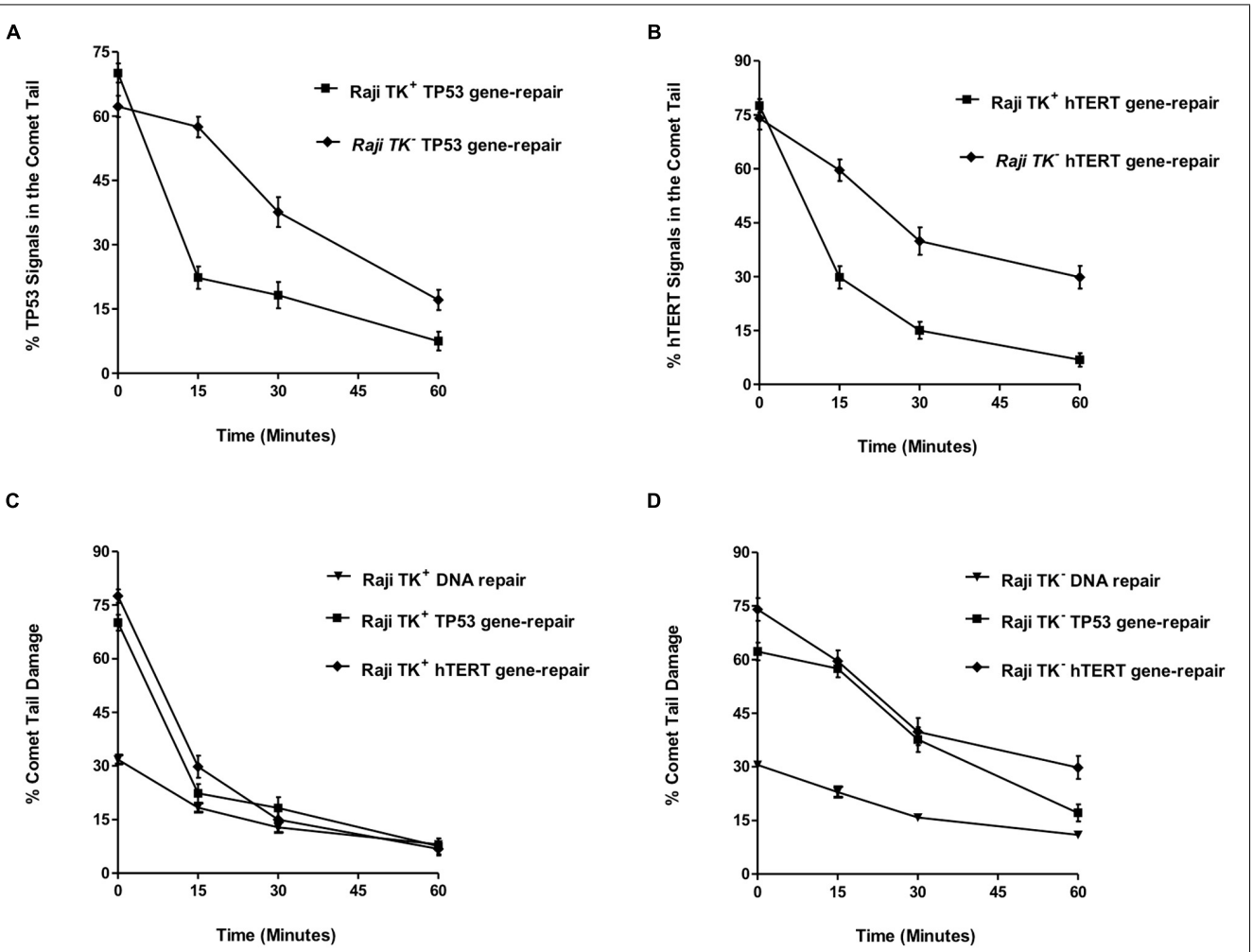


FIGURE 4 | TP53 and hTERT gene-repair in TK1$^-$ and TK1$^+$ cells and comparison with the overall genome. (A) shows the % TP53 tail signals and **(B)** the % hTERT tail signals over the repair time period after 5 Gy-irradiation. **(C,D)** compares DNA repair between the overall genome and specific gene-regions. These findings demonstrate that preferential repair is occurring in the TP53 and hTERT domains of Raji TK1$^+$ but not in TK1$^-$ cells. In each graph, fifty cells were analyzed at each time-point for each experimental replicate. Each data point represents the mean ± SEM of three independent replicate experiments.

We confirmed that TP53 and hTERT are substrates for preferential repair of gene sequences repair in Raji cells – intrinsically probable, since TP53 is transcribed throughout the cell cycle (Liu and Chen, 2006) and further induced by DNA-damaging agents, while the hTERT gene is upregulated in malignant cells such as Raji (Ducrest et al., 2002). RTPCR analysis found that both TK1$^+$ and TK1$^-$ clones actively expressed the hTERT and TP53 genes. Also, since the correct function of the TP53 gene product is a major factor in mediating DNA repair processes (reviewed by McKay et al., 1999) it is possible that disparity in DNA repair between TK1$^+$ and TK1$^-$ cells could arise if there were different TP53 mutations in either cell-line. Sequencing results for both TK1$^+$ and TK1$^-$ clones confirmed that the TK1$^+$and TK1$^-$ clones have the same TP53 mutations and should therefore have the same tumor suppressor protein function during DNA repair.

The Comet-FISH data show that although TK deficiency does not prevent the repair of strand breaks in bulk DNA, it is slower in the TK1 deficient cell-line. The slight deceleration in bulk DNA repair in TK1$^-$ is likely a reflection of the markedly reduced repair occurring in damaged regions containing transcribed genes. Our data shows that Raji TK1$^-$ cells display poor initial repair of regions containing transcribed genes (as evidenced by a slower reduction of TP53 and hTERT tail hybridization signals). The rapid TP53 gene region repair demonstrated in TK1$^+$ cells is also consistent with other studies of repair in this gene (McKenna et al., 2003; Horvathova et al., 2004). These results clearly demonstrate that TK1 is necessary for the rapid repair of both the TP53 and hTERT gene region.

One possibility to explain the difference in preferential repair between TK1$^+$ and TK1$^-$ clones is that TK1 provides a dTTP pool that facilitates the rapid repair of damaged genes. Raji TK1$^+$ cells were therefore cultured in thymidine free media to deprive TK of its substrate, and damage induced gene

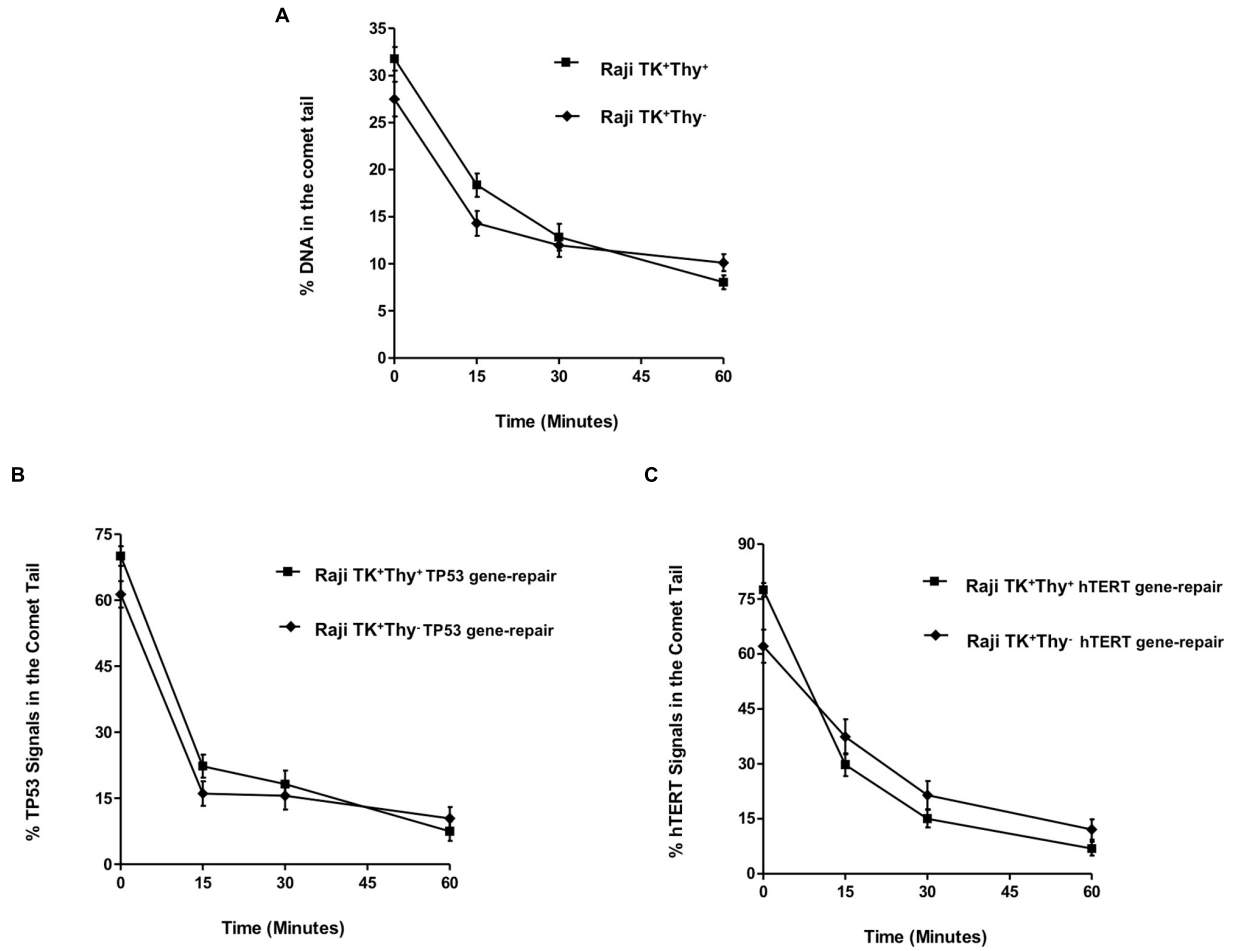


FIGURE 5 | Effect of thymidine salvage depletion in Raji TK1$^+$ cells. **(A)** depicts the repair of bulk DNA, while graphs **(B,C)** show the % TP53 and hTERT tail signals over the repair time period after 5 Gy-irradiation. The rapid gene repair observed clearly demonstrates that culturing of TK1$^+$ cells in thymidine free media has no effect on the level of preferential repair. Fifty cells were analyzed at each time-point for each experimental replicate. Each data point represents the mean ± SEM of three independent replicate experiments.

region specific and bulk DNA repair were assessed. The results showed that the preferential repair capabilities of TK1$^+$ cells were unaffected: therefore TK1 does not exert its effects through deoxyribonucleotide pools, otherwise repair of TK1$^+$ cells would have been rendered similar to that of repair deficient TK1$^-$ cells.

We have thus demonstrated that TK1 is a novel factor regulating preferential, gene-specific repair, operating not through its enzymic role but by some other mechanism: possibly activation of another protein involved in the repair process. There is no current evidence for direct interaction of TK1 protein with any known component of the single-strand break repair pathway. If one were to speculate, one might propose as a possible candidate for direct or indirect dependence on TK protein the DNA 3′phosphatase, in some circumstances a rate-limiting factor in single-strand break repair (Breslin and Caldecott, 2009). We note that defects in TK are well established as being mutagenic, and hence they are presumably also carcinogenic.

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Controlling variation in the comet assay

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Variability of the comet assay is a serious issue, whether it occurs from experiment to experiment in the same laboratory, or between different laboratories analysing identical samples. Do we have to live with high variability, just because the comet assay is a biological assay rather than analytical chemistry? Numerous attempts have been made to limit variability by standardizing the assay protocol, and the critical steps in the assay have been identified; agarose concentration, duration of alkaline incubation, and electrophoresis conditions (time, temperature, and voltage gradient) are particularly important. Even when these are controlled, variation seems to be inevitable. It is helpful to include in experiments reference standards, i.e., cells with a known amount of specific damage to the DNA. They can be aliquots frozen from a single large batch of cells, either untreated (negative controls) or treated with, for example, H_2O_2 or X-rays to induce strand breaks (positive control for the basic assay), or photosensitiser plus light to oxidize guanine (positive control for Fpg- or OGG1-sensitive sites). Reference standards are especially valuable when performing a series of experiments over a long period—for example, analysing samples of white blood cells from a large human biomonitoring trial—to check that the assay is performing consistently, and to identify anomalous results necessitating a repeat experiment. The reference values of tail intensity can also be used to iron out small variations occurring from day to day. We present examples of the use of reference standards in human trials, both within one laboratory and between different laboratories, and describe procedures that can be used to control variation.

Keywords: comet assay, variability, reference standards, biomonitoring, inter-laboratory comparisons

INTRODUCTION

The comet assay is conventionally seen by many as a soft, biological assay, at best semi-quantitative. Variation does occur, between laboratories, and over time in the same laboratory, and so some form of standardization is advisable. It is in principle possible to improve the comparability of comet data by expressing results not just as % tail DNA, but as a frequency of DNA breaks, by calibrating the assay using cells that have been treated with different doses of X- or γ-radiation; it has been known since the days of alkaline sucrose gradient sedimentation that ionizing radiation induces damage in cellular DNA at the rate of 0.31 breaks per Gy per 10^9 Dalton (Ahnstrom and Erixon, 1981). (Ionizing radiation is a very robust damaging agent, compared with chemicals, which can be greatly affected by the physico-chemical conditions of exposure, and particularly by the biological environment of enzymes and other molecules that can react with the chemical, decreasing or increasing its effectiveness—not to mention possible membrane barriers that can limit uptake.) This conversion to a "real" break frequency is not without its problems: most researchers do not have access to a radiation source, and so calibration tends to be second-hand, and historical. Even if a source is available, so that irradiated reference cells can be included in the same experiment, these cells and the sample cells are never assayed under the exact same conditions; they cannot be together in the same gel (as a true internal standard would be) unless some way is found to distinguish the two cell types after electrophoresis. (There are attempts to overcome this difficulty, as discussed later in this review.)

The comet assay is widely used in combination with formamidopyrimidine DNA glycosylase (Fpg) or 8-oxoGua DNA glycosylase (OGG1) to measure 8-oxoGua in DNA—an excellent marker of oxidative stress. In the mid-1990s, it became clear that estimates of the background level of 8-oxoGua in peripheral blood mononuclear (PBMN) cells from healthy subjects varied by orders of magnitude, depending on the assay employed. The comet assay + Fpg gave results on the low side, compared with chromatographic methods (HPLC, LC-MS.MS, GC-MS). In the ESCODD project (ESCODD, 2002, 2003; ESCODD et al., 2005) we set out to compare the different methods and decide which was most accurate. It turned out that the chromatographic methods were subject to oxidation of DNA during sample preparation, leading to a serious over-estimation of DNA base oxidation. The comet assay was free of this artifact. However, while the comet assay + Fpg was apparently more accurate than chromatography, it suffered—and still suffers—from lack of precision. ESCODD partners were sent identical cell samples to analyse, but the results varied greatly from laboratory to laboratory. By analogy with a

game of darts, the comet assay results were clustered around the bull's eye, but did not score direct hits.

Here we will discuss the different levels of variation—experimental, inter-laboratory, intra-individual, inter-individual (or between different samples—e.g., different concentration of a chemical compound—in the case of experiments with cell culture), and even inter-national—that can be encountered with the comet assay. It is important to recognize—and limit as much as possible—the variation that arises from differences in experimental conditions, in order to maximize the variations that are of real interest, e.g., the variations between samples, subjects in a population study, or population groups in different countries. We will describe the measures that should be taken to ensure experimental consistency, encourage the use of reference standards, and suggest ways of accommodating experimental variation by normalization procedures.

IDENTIFYING SOURCES OF VARIATION

Trials have been carried out, in particular by the consortium known as ECVAG, specifically to examine variability in the comet assay, and to apply statistical analyses to quantitate the different sources of variation.

In the first ECVAG trial (Forchhammer et al., 2010), 12 laboratories received pre-made slides to score, a set of cryopreserved γ-irradiated cells to construct a standard curve, and a set of coded samples. The inter-laboratory coefficient of variation (CV) for the latter, 47%, was reduced to 28% when data were adjusted using the laboratory-specific standard curve. The second trial (Johansson et al., 2010) involved 10 laboratories and examined variation in the measurement of Fpg-sensitive sites; coded samples treated with Ro 19-8022 plus light were sent with a set of γ-irradiated reference samples. The inter-laboratory variation in assessment of Fpg-sensitive sites was mainly due to differences in protocols, and was decreased when standard curves (created from the reference samples) were used to adjust the results. The aim of the third ECVAG trial (Forchhammer et al., 2012) was to test the effect of introducing a standard protocol; several laboratories found it difficult to adopt the standard methods. Three coded human PBMN cell samples were analyzed: variation was very high, for strand breaks, and for Fpg-sensitive sites, and (in the case of Fpg-sensitive sites) was only slightly less with the standard protocol than with the laboratories' own protocols. The fourth trial (Ersson et al., 2013) set out to identify different sources of variation in analysis of real PBMN cell samples, and concluded that "inter-laboratory variation accounted for the largest fraction of the overall variation and the unexplained (residual) variation was much larger than the intra-laboratory variation. . ."

LIMITING VARIATION

Differences in protocol seem to be largely responsible for variation between laboratories. Some likely sources of variation are obvious, but still deserve to be formally explored, and this was done independently by two groups a few years ago, with very similar conclusions. Ersson and Möller (2011) and Azqueta et al. (2011a) investigated the effect on comet formation (% tail DNA) of agarose concentration, duration of alkaline unwinding, electrophoresis period and voltage gradient. The % tail DNA of comets from cells treated with γ-rays (Ersson and Möller, 2011) or with H_2O_2 (Azqueta et al., 2011a) was greatest in 0.4% agarose (which is fragile, and not recommended), and steadily decreased with increasing concentration, up to >1%. The period of alkaline incubation before electrophoresis was varied, in both laboratories, up to 60 min, and steady increases in % tail DNA with time (at least up to 40 min) were seen in comets from cells treated with H_2O_2 (both laboratories) and also γ-irradiated cells and cells treated with photosensitiser plus light (to induce 8-oxoGua) and incubated after lysis with Fpg (Ersson and Möller, 2011). A similar dependence on time of alkaline unwinding was previously shown by Vijayalaxmi et al. (1992) and Speit et al. (1999). When the alkaline unwinding period was extended to 18 h, all DNA (from cells treated with N-methyl-N-nitro-N-nitrosoguanidine) was present in the tail (Yendle et al., 1997). The increase in tail intensity is likely to be due to conversion of alkali-labile sites (such as result from loss of bases from the DNA) to strand breaks.

It is generally assumed that the initial lysis in high salt and detergent is not critical, and lysis periods of 1 h, or overnight, or even days, or weeks, are common. A recent study (Enciso et al., in press) found that, for cells treated with methylmethanesulphonate or H_2O_2, similar values of % tail DNA were seen with 1 h of lysis or with no lysis at all (i.e., immediately placing slides into alkaline solution). Longer lysis periods, up to 1 week, led to an increase in % tail DNA, in the case of treated cells. However, if enzyme digestion is included in the comet assay procedure, for instance to detect 8-oxoGua, lysis is essential to make the DNA accessible to the enzyme; in this case, 5 min or 1 h of lysis gave similar results (Enciso et al., in press). Sensitivity (i.e., relative increase in % tail DNA in treated compared with untreated cells) was enhanced up to 24 h.

For comets from irradiated or H_2O_2-treated cells, the % tail DNA is strongly influenced by both electrophoresis time (varied up to 40 min) and voltage gradient (between <0.2 and 1.6 V/cm) (Ersson and Möller, 2011; Azqueta et al., 2011a) (**Figure 1**). To a certain extent, a low voltage gradient for a long time will give similar results to a higher voltage gradient for a shorter time. The voltage gradient should be measured over the platform on which the slides are placed rather than between the electrodes, since that is where the electric potential pulls out damaged DNA from the nucleoids. Between the electrode and platform edge, in standard tanks, there is a relatively deep trough of electrophoresis solution, with low resistance, so that the voltage drop is much lower than over the platform where there is a shallow layer of solution over the slides; hence, measuring the total applied voltage and dividing it by the distance between the electrodes gives an erroneous V/cm value. Increasing the depth of solution over the platform increases the current (because the resistance is decreased), and this causes a slight decrease in the % tail DNA—an effect explained by the reduced voltage drop over the platform (Azqueta et al., 2011a). Current itself does not influence DNA migration. These comments relate to tanks with electrodes in troughs and a central platform. There are few restrictions in tank design as long as the voltage gradient is constant where the samples are placed for electrophoresis. We recommend careful

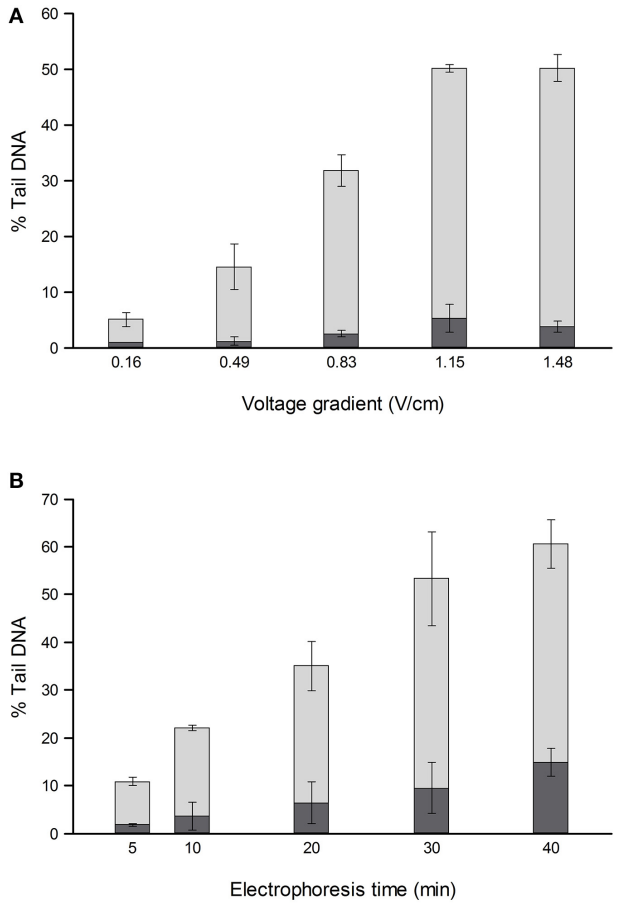

FIGURE 1 | Effect of different voltage gradients at constant current (A) and different electrophoresis times (B) on the % tail DNA in TK-6 cells, untreated (dark shading), and treated with 70 μM H_2O_2 (light shading). The mean and range of values from two experiments are shown. Redrawn from Azqueta et al. (2011a) with permission from Elsevier.

measurement of the voltage gradient at the relevant position and depth, particularly when non-standard electrophoresis tanks are being used.

Ersson and Möller (2011) looked also at the enzyme incubation step, measuring 8-oxoGua induced by photosensitiser Ro 19-8022 plus light. At a specific enzyme concentration, a maximum yield of DNA breaks (% tail DNA) was seen after 30 min digestion, with no increase at 45 min. This simply highlights the necessity to optimize incubation conditions for each batch of enzyme, whether obtained commercially or prepared in-house from an overproducing bacterial strain. It should also be noted that, since enzyme kinetics depend on affinity for substrate, if Fpg is used to detect lesions other than 8-oxoGua, optimal enzyme concentration/incubation time may differ, and should be separately determined. The results of these studies suggest that variation within a laboratory is reduced if care is taken to control these critical parameters. We can recommend conditions, within limits: 0.6-0.8 % agarose (final concentration), 40 min alkaline incubation, and electrophoresis for between 20 and 30 min at around 1 V/cm. Whichever conditions are chosen, they should be precisely maintained, and reported in publications, to facilitate comparison between laboratories.

Within an electrophoresis run, there can be variation, depending on the position of the gel on the platform. This is likely due to local variations in voltage, which are detected by placing a measuring gauge with platinum probes at defined height and spacing on the platform. The variations in voltage—and the variation in % tail DNA of ostensibly identical cell samples—were considerably reduced by introducing mild recirculation of electrophoresis solution using an external pump (Gutzkow et al., 2013).

It is generally recommended that electrophoresis be carried out under refrigeration, so that the temperature of the solution, and the gels, does not rise above 15°C. McKelvey-Martin et al. (1993) showed that, for γ-irradiated and unirradiated lymphocytes, there was little difference in comet appearance with alkaline incubation and electrophoresis at 5°C or 10°C, but a substantial increase in migration occurred at higher temperatures (up to 25°C). This was confirmed by Speit et al. (1999) comparing 4°C and 20°C and was recently also reported by Sirota et al. (2014). Speit et al. (1999) suggest that the higher temperature might be usefully employed to increase the sensitivity of the assay. In any case, a tank with efficient temperature control and monitoring would be a reassuring technical advance.

What level of variation is acceptable? During the recent COMICS project, two partner laboratories carried out similar experiments with TK-6 lymphoblastoid cells treated with 0.25 mM methylmethanesulphonate for 3 h, using exactly the same protocol (Azqueta et al., 2013). We were testing different formats; the standard format of two large gels per slide, or 12 mini-gels per slide, or minigels in an 8 × 12 multi-array format. The CVs were calculated for replicate gels in each of three experiments in each laboratory, and the mean CV was then calculated. **Table 1** shows that, for no apparent reason, one laboratory had more variable results than the other. Combining the results from the two laboratories gave CVs of just over 10%—essentially the same for all three formats. Testing the 8 × 12 multiarray format, with X-irradiated cells, Gutzkow et al. (2013) reported a mean CV from three experiments of 26% without recirculation of electrophoresis solution, which reduced to only 7% with circulation. We conclude that a CV of around 10%, for identical cell samples with appreciable damage levels, would be a realistic target (At very low levels of damage, close to 0% tail DNA, the CV will of course be much higher and is no longer meaningful. Conversely, at high levels of damage, as saturation of the assay is approached, the CV will tend to be very small, but again this has little meaning).

REFERENCE STANDARDS

It is sound practice to include reference standard cells in experiments. They should be from a single batch of, for example, human PNMN cells or cultured cells, either untreated (negative controls), or treated with DNA-damaging agent relevant for the particular experiment. Aliquots are then stored under conditions preserving DNA integrity. Slow freezing of PBMN or cultured cells in medium containing serum and 10% dimethylsulphoxide (DMSO) prevents physical damage to DNA; aliquots should

Table 1 | Coefficients of variation.

Format	Laboratory A (%)	Laboratory B (%)	Combined (%)
2 Gels/slide	4.8	15	12
12 Minigels/slide	7.5	16	12
24 Minigels/GelBond	8.2	13	11

TK-6 cells were treated with 0.25 mM MMS for 3 h. The comet assay was performed using different formats, in two laboratories. The CV was calculated for each of 3 independent experiments in each laboratory; the mean CV is shown here. Also shown is the mean CV for all experiments in both laboratories combined. Data from Azqueta et al. (2013).

be thawed quickly, diluted with PBS or medium and centrifuged without delay to remove the cells from DMSO.

The reference standards serve to monitor performance of the assay. If a particular experiment gives seriously anomalous results for the standards, the results for the samples should be scrutinized, and if necessary the experiment should be repeated. However, minor variations are inevitable, and it is possible to use the reference standard % tail DNA results to improve the precision of sample results.

Standards are particularly important when many samples (for example, from a human biomonitoring trial) are analyzed in a series of experiments over an extended period. **Figure 2** shows typical results from such a trial; standards—either untreated, or treated with Ro 19-8022 plus light to induce 8-oxoGua—were included in each experiment. The CV for the untreated cells was 52%, while for the treated cells it was 14%. Variation in experiments carried out over a long time period can be expected to be greater than variation within an experiment. **Figure 2** also illustrates the point made above, that where levels of damage are close to the limit of detection (0% tail DNA), the relative variation will be greater.

Ideally, reference standards would be internal standards, i.e., cells embedded in the same gel as the sample cells. The problem of distinguishing standard cells from sample cells after electrophoresis has been solved in more than one way, although to the best of our knowledge true internal standards are not employed routinely in any comet assay laboratories. One solution is to pre-label standard cells by incubating them over a cell cycle with bromodeoxyuridine, which is incorporated into DNA in place of thymidine. It can subsequently be recognized by means of a fluorescent-tagged anti-bromouracil antibody (Zainol et al., 2009). When scoring, appropriate (different) filters are used to identify sample and standard cells, and this makes the process of scoring more laborious. A second approach uses as standards cells with a markedly different genome size compared with human—for example, erythrocytes of certain fish species (Brunborg et al., 2014). After scoring all the comets in the gel, they are sorted into two sets according to total comet fluorescence, which is proportional to genome size.

NORMALIZATION

We suggest a procedure for correcting sample data for experimental variation as revealed by reference standards in a series of experiments. As an example, we assume that sample cells have been analyzed for 8-oxoGua (Fpg-sensitive sites) and that data are available from reference standards treated with Ro 19-8022 plus light to induce 8-oxoGua (Positive reference standards should be used; comets from untreated cells are too much affected by high relative variation to be useful for normalization).

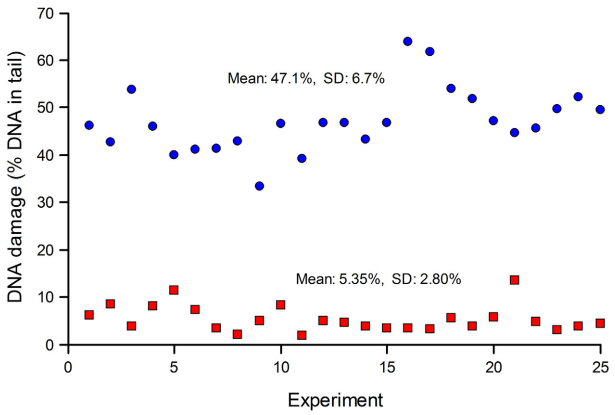

FIGURE 2 | DNA damage in reference standards, assayed in a series of experiments to measure DNA damage in PBMN cells from a human biomonitoring study. Aliquots of human lymphocytes from a single batch, either untreated (red squares), or treated with Ro 19-8022 to induce 8-oxoGua (blue circles), were included as standards in each of the experiments alongside the test samples (the results of which are not shown) and analyzed for strand breaks and Fpg-sensitive sites, respectively.

- Calculate the median value, M, of net Fpg-sensitive sites (% tail DNA) for the reference cells in all experiments in the series. (Taking the median excludes the anomalously high or low values.)
- With the value of net Fpg-sites (% tail DNA) for the reference cells in a particular experiment X defined as Q, then the correction factor is M/Q.
- Multiply the values of Fpg-sites (mean or median % tail DNA) for samples in experiment X by M/Q.

Figure 3 gives an example of normalization. Samples of lymphocytes from an intervention study were analyzed for Fpg-sensitive sites. The data were then corrected for variation as indicated by positive reference standards run in the same experiments. In most cases, normalization made little difference, but substantial changes were seen in a few samples, namely 5 and 9 in this set of samples.

TRUE VARIATION

Figure 3 gives a good idea of the range of DNA damage levels (in this case, oxidized purines) to be found in an apparently healthy population. In the ESCODD project (ESCODD et al., 2005), we attempted to answer the question whether there are significant differences in DNA damage levels between countries (**Figure 4**). Partners in this project were asked to collect PBMN cells from healthy volunteers and to measure Fpg-sensitive sites. They also measured Fpg-sites in standard samples of HeLa cells containing

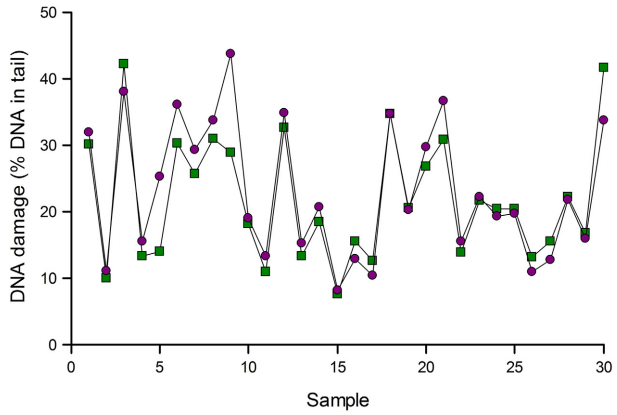

FIGURE 3 | Normalization of comet assay data. Results of analysis of 30 lymphocyte samples using Fpg to detect 8-oxoGua were corrected for variation as indicated by reference standards (see text). Data are shown before (green squares) and after (purple circles) normalization. Results for samples 5 and 9 changed substantially after normalization.

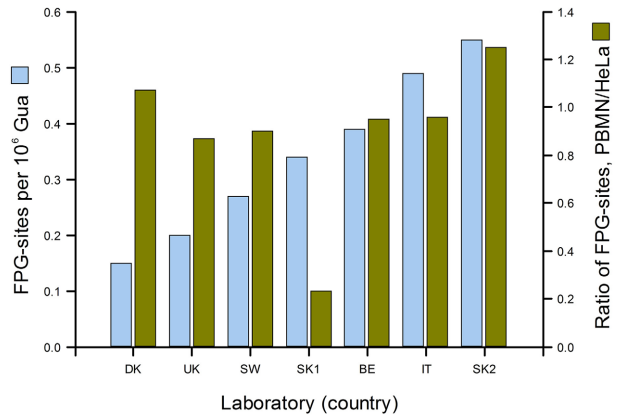

FIGURE 4 | DNA damage levels in PBMN cells from representative groups of between 8 and 20 healthy subjects in Denmark, United Kingdom, Sweden, Slovakia (two laboratories), Belgium and Italy. Mean % tail DNA for each laboratory was converted to Fpg-sensitive sites per 10^6 Gua (light blue bars) using an X-ray calibration curve. To correct for variation between laboratories, mean % tail DNA for PBMN cells was divided by mean % tail DNA for standard HeLa cells (treated in the coordinating laboratory with Ro 19-8022 plus light, and distributed frozen to the partners). The corrected values are shown in dark green (From Collins, 2014 with permission from Elsevier).

8-oxoGua induced by Ro 19-8022 plus light. The CV of the mean values from the seven laboratories was 43%. When the means were corrected for inter-laboratory variation, by dividing PBMN cell means by the value found for HeLa cells in each laboratory, there was much less variation among the countries—with one exception, which gave a very low value. (This happened to be one of the two laboratories from one country; the other laboratory had a result closer to those of the other countries, and so we assumed that a technical problem in the first laboratory accounted for the low value.) Omitting this outlier, the CV for mean damage levels was only 14%. We can conclude that, in this sample of six countries from different corners of Europe levels of oxidative damage to DNA were quite uniform.

CONCLUSIONS

It is now clearer than ever what are the experimental conditions that most critically influence the % tail DNA recorded for a given cell sample: agarose concentration, electrophoresis time and voltage gradient. For each of these, the effect of variation over a fairly wide range of values is more or less linear. Other factors—lysis time, alkaline incubation time, enzyme concentration and incubation time, electrophoresis temperature—are also important, but optimal conditions can be established which allow a certain amount of latitude; thus, for example, if all enzyme-sensitive sites are detected in 30 min, extending incubation to 45 or 60 min should have no effect. While it is unreasonable to expect all laboratories to adopt exactly the same conditions, they should (a) ensure that whatever conditions are chosen are precisely maintained from experiment to experiment, and (b) specifically describe those conditions in any publication (even though for the overall procedure reference may be made to a previous publication).

The "elephant in the room" is the issue of staining, scoring and image analysis. A conclusion from the first ECVAG trial (Forchhammer et al., 2010) was that most of the observed inter-laboratory variation results from different procedures in staining and analyzing comet images. The concentration of stain can influence comet assay results, as was shown by Olive et al. (1990) in the case of propidium iodide. A wide range of different stains are in use, and little effort has been made to check whether they give comparable results. Comparing different staining procedures, the intercalating dye propidium iodide, minor groove-binding Hoechst 33342 and DAPI showed similar sensitivities (indicated by the slopes of dose-response curves), as did bromodeoxyuridine incorporated into replicating DNA and detected with FITC-conjugated anti-BrdUrd (Olive et al., 1992). The traditional UV light source (mercury vapor lamp) varies in output over time; modern LED light sources are more stable. Various scoring systems are in use. Visual scoring simply categorizes comets into classes [typically from "no tail" (class 0) to "almost all DNA in tail" (class 4)] and computes the overall score for 100 comets, between 0 and 400 arbitrary units. Image analysis, based on a variety of commercial or free software systems, computes mean % tail DNA, tail moment, tail length and other more abstruse properties; most commonly used are % tail DNA and tail moment. (The issue of which parameter to use is addressed in a separate article, by Møller et al., 2014.) Image analysis systems can be manual (i.e., comets being selected by the operator for analysis) or automated. Visual scoring, manual and automated image analysis were compared (Azqueta et al., 2011b), they gave qualitatively similar results in dose response experiments with MMS and H_2O_2, but visual scoring overestimated low levels of damage while automated analysis missed highly damaged comets—a defect since rectified.

Sadly, numerous inter-laboratory trials by ESCODD and ECVAG have failed to eliminate variability from the comet assay. There is a need for further ring studies, with even more strictly

controlled experimental conditions, distribution of cell samples with different levels of damage, analysis of PBMN cells prepared locally and subjected to defined doses of ionizing radiation, and exchange of the resulting slides between laboratories for re-scoring with different systems—something that has not been done systematically before.

Within a laboratory, experimental variation of around 10% is acceptable, though this will depend on the damaging agent: cells treated with ionizing radiation are likely to show damage responses that are more homogeneous than when treated with chemicals, since cellular metabolic responses are not involved, and so the variation between gels or samples should be relatively low.

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Reference cells and ploidy in the comet assay

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In the comet assay single cells are analyzed with respect to their level of DNA damage. Discrimination of the individual cell or cell type based on DNA content, with concomitant scoring of the DNA damage, is useful since this may allow analysis of mixtures of cells. Different cells can then be characterized based on their ploidy, cell cycle stage, or genome size. We here describe two applications of such a cell type-specific comet assay: (i) Testicular cell suspensions, analyzed on the basis of their ploidy during spermatogenesis; and (ii) reference cells in the form of fish erythrocytes which can be included as internal standards to correct for inter-assay variations. With standard fluorochromes used in the comet assay, the total staining signal from each cell – whether damaged or undamaged – was found to be associated with the cell's DNA content. Analysis of the fluorescence intensity of single cells is straightforward since these data are available in scoring systems based on image analysis. The analysis of testicular cell suspensions provides information on cell type specific composition, susceptibility to genotoxicants, and DNA repair. Internal reference cells, either untreated or carrying defined numbers of lesions induced by ionizing radiation, are useful for investigation of experimental factors that can cause variation in comet assay results, and for routine inclusion in experiments to facilitate standardization of methods, and comparison of comet assay data obtained in different experiments or in different laboratories. They can also be used – in combination with a reference curve – to quantify the DNA lesions induced by a certain treatment. Fish cells of a range of genome sizes, both greater and smaller than human, are suitable for this purpose, and they are inexpensive.

Keywords: comet assay, genome size, testicular cells, fish cells, reference cells

INTRODUCTION

In our past studies of genotoxicity in mixtures of primary cultures of testicular cells from rats and humans (Bjorge et al., 1996a), there was a need to characterize subpopulations of spermatogenic cells. Flow cytometric analysis of DNA-stained cells was used for this purpose. We applied alkaline filter elution to measure DNA damage in testicular cell populations partly purified by means of centrifugal elutriation. We subsequently found that different testicular cell types could be identified when using the comet assay, which – unlike alkaline elution – allows measurement of DNA damage in individual cells (Bjorge et al., 1996b). This was possible since the fluorescent signal in the comet assay is related to the amount of DNA, which depends on cell ploidy. Testicular cell suspensions contain spermatogonia and secondary spermatocytes (but also Sertoli and Leydig somatic cells; 2n), primary spermatocytes (4c), secondary spermatocytes after first meiotic cleavage (2C), and spermatids and spermatozoa at different stages of differentiation and maturation (1n). The relative proportions of these cell types are specific for human and rat testicular cells (Bjorge et al., 1996a; Olsen et al., 2003). Provided that scoring conditions (light intensity and staining) are standardized, the response of cell populations may be compared in different comet assay experiments. We used these approaches to estimate DNA damage induction and its repair in spermatogenic cells from mixed testicular cell populations (Olsen et al., 2001, 2003).

Cell-specific fluorescence can also be used as a basis for reference cells in the comet assay, as will be shown here. Reference cells are useful in standardization of conditions during various stages of the experimental protocol. Unexpected variations in measurement of specific DNA lesions occur between laboratories (Forchhammer et al., 2010), even for experienced comet assay users. Calibration trials, validation efforts, standardization of methods, and comparison of comet results between experiments and laboratories should profit from reference cells which could be analyzed in parallel with the sample cells. Reference cells could be in neighboring gel samples, or – preferably – mixed with the samples cells before the comet analysis and therefore subjected to exactly the same treatment conditions at all steps of the comet assay protocol (the only requirement being that the reference comets should be distinguishable from the comets from sample cells). This should also allow a better control of local variations in electrophoresis conditions. Zainol et al. (2009) developed a comet assay using internal bromodeoxyuridine-prelabelled reference cells that were identified on the basis of *in situ* immunostaining before scoring. Such cells can be mixed with unlabelled cells. This very useful approach, however, involves extra treatment steps and – compared with a method based on differential DNA content – is more costly because the method relies on antibodies.

We describe how DNA content-specific fluorescence intensity in the comet assay can be used, (i) for characterization of testicular cell populations; and (ii) as a convenient and low-cost system for

internal reference cells taking advantage of the lower DNA content of some species of fish.

MATERIALS AND METHODS

Human testicular biopsies were obtained from organ donors, and single-cell suspensions of testicular cells were prepared as described (Bjorge et al., 1996a). Cells (unfrozen) were processed for comet assay analysis, stained with ethidium bromide, and analyzed using the Fenestra Comet image analysis system (Kinetic Imaging LTD, Liverpool, UK; Bjorge et al., 1996b). Comet Tail Moments (TM; these experiments were partly done in the early days of the comet assay, when TM was often used) and total fluorescence intensity (TFI) were recorded for each cell; these parameters are already integrated in Fenestra. During scoring no cells were excluded on the basis of either a very strong or a very weak fluorescence signal.

Blood (1–2 mL) was drawn using a 2 mL syringe from the tail vein of a turbot (*Scophthalmus maximus*) weighing about 600 g, kept in an indoor aquarium (about 50 m^3, 8°C, continuously flushed with fresh sea water from the Oslo fjord at Solbergstrand, Drøbak, 40 km south of Oslo). The blood was diluted 1:10 in ice-cold phosphate buffered saline (PBS; 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl) with 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4; or in RPMI1640 medium (w/Hepes and glutamine; pH 7.4); and transported on ice to the laboratory in Oslo. After microscopic examination and counting, samples of the fish erythrocytes (FE), which are nucleated, were diluted once more 1:10 in RPMI1640 but now containing 10% dimethyl sulfoxide (DMSO) and 20% foetal calf serum (FCS), and 1 mL aliquots were frozen slowly to −80°C while placed in a Mister Frosty™ Freezing Container (Thermo Scientific, Oslo, Norway) unit containing isopropanol, for slow freezing of biological samples (the temperature is reduced at a rate of approximately 1° per minute). For use, samples were thawed by warming for a few seconds to allow the frozen ice to be transferred into a tube containing 10 mL RPMI medium followed by centrifugation for 10 min at 400 ×*g* and washing in the same medium. Suitable aliquots were mixed 1:10 in LMP agarose (0.7%) and analyzed according to our protocol for 96 minigels and electrophoresis with circulation (Gutzkow et al., 2013). Fresh (unfrozen) samples were diluted in RPMI medium (without DMSO/serum), mixed with agarose, and analyzed in the same way. The FE were added to GelBond® films either as independent samples, or they were mixed 1:1 with human peripheral blood mononuclear cells (PBMN), which had been previously frozen, before embedding in agarose on GelBond® films (for details, see Gutzkow et al., 2013). Photomicrographs of stained cell samples were recorded using Comet IV (see below). In some experiments, samples were irradiated before analysis with defined doses of X-rays (260 KeV, filtered through 0.5 mm Cu, dose rate 10 G/min) on ice.

For scoring of FE and PBMN comets, dried GelBond films® were rehydrated and stained with SYBRGold (Life Technologies Ltd, Paisley, UK; 2 μL in 25 mL TE buffer pH 7.4, 20 min at RT), rinsed in water, and analyzed with Comet IV (Perceptive Instruments Ltd, Bury St. Edmunds, UK). An Olympus BX51 fluorescence microscope with CCD camera was used. Tail%DNA (TD) and TFI were recorded for each cell; these parameters are already integrated in Comet IV. During scoring no cells were excluded on the basis of either a very strong or a very weak fluorescence signal.

Statistics: Means and frequency distributions were calculated using Microsoft Excel.

RESULTS

COMET ANALYSIS OF TESTICULAR CELL SUSPENSIONS

The results shown in **Figure 1** were obtained by pooling all data from an analysis of 16 samples of unexposed (control) fresh human testicular cells (from one donor). The data were sorted according to TFI values and they fell into three distinct classes. Based on the distribution of the total intensities, thresholds were sought for separation of the three populations of cells (vertical lines in **Figure 1**). The profile resembles the graphs obtained with flow cytometric analysis of testicular cells (Bjorge et al., 1996b). The mean fluorescence intensities (≈ DNA content) are given in the legend. These cells were not subjected to genotoxic treatment and the levels of DNA damage in the three classes were low and very similar.

REFERENCE CELLS FROM FISH WITH LOW GENOME SIZE

In initial experiments, we tried fish cells from various genera. The polar cod, *Boreogadus saida*, has a suitable genome size (0.88 pg haploid genome; http://www.genomesize.com/), but the intrinsic level of DNA damage in frozen cells was high and variable. Rainbow trout (*Oncorhynchus mykiss*) had less DNA damage but its genome size (2.4–2.7 pg) is quite similar to that of *Homo sapiens* (3.50 pg); this was obvious in scatter plots similar to **Figure 3** (see below) but without the two distinct populations (data not shown). In the subsequent experiments we used the turbot (*Scophthalmus maximus;* 0.86 pg).

Figure 2 illustrates the appearance in fluorescence microscopy of the different control (unexposed) cells of HPBL and FE, embedded either in separate gels or mixed 1:1 in the same gel before analysis with the comet assay. As with the testicular cells, the mixed cell population of control cells (HPBL and FE) belong to distinct classes but in this case there are only two classes (**Figure 3A**).

The mean TFI for turbot FE in **Figure 3A** is approximately 3–4 times lower than for HPBL, as expected from the genome size differences (see also **Figure 4**). It is apparent from the figure that there are no cells with TFI between 70,000 and 100,000. The absolute threshold value is subject to inter-experimental variations depending on the intensity of the lamp and the staining of DNA. Traditional fluorescent light sources (Mercury Xenon) produce less light with time of use, but newer technologies (liquid light guide combined with metal halide or LED light) solve this latter problem.

Other FE and HPBL samples were exposed to of X-rays (0, 1.5, 3, 6, 8, 10, or 15 Gy) on ice and analyzed quickly to prevent repair of DNA strand breaks. The graphs in **Figure 3** show scatter plots of DNA damage (TD) of the cell populations. The TD values may be discriminated and analyzed separately for the two populations (i.e., intensity levels either below 70,000 or above 100,000). Mean TD values were calculated for each population in **Figure 3** and used to construct the dose response curves for both cell types presented in **Figure 4**. DNA damage increases linearly with the radiation

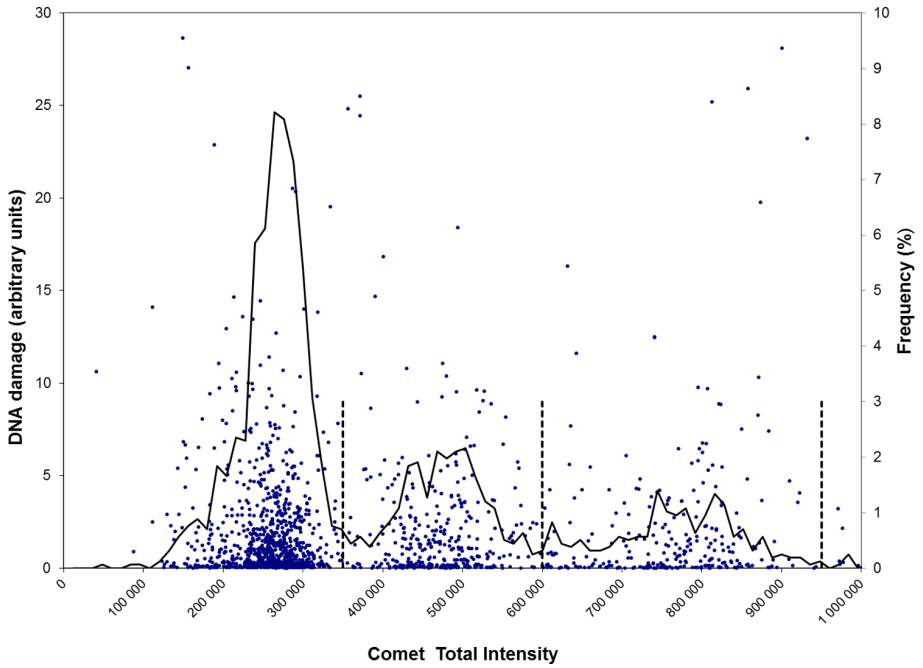

FIGURE 1 | Scatter plots of DNA damage of cells of different ploidy. Each single cell (with varying tail size) is scored for DNA damage together with its total fluorescence intensity (TFI). DNA damage (left vertical axis) and TFI (horizontal axis) were determined for 1600 control cells (dots) prepared from a human testicular biopsy. The stippled vertical lines indicate the partitions of three different populations of cells, of ploidy 1n, 2n, and 4c (discriminated at fluorescence intensity 350 000, 600 000, and 950 000, respectively). The mean intensities of cells within these classes are 255 000, 471 000, and 761 000 for 1n, 2n, and 4c, respectively. Mean levels of DNA damage (TM) are in the range 2.2–2.5 for the three classes and are not significantly different. Plotted line: mean frequency (%, right vertical axis) of DNA damage at each intensity level.

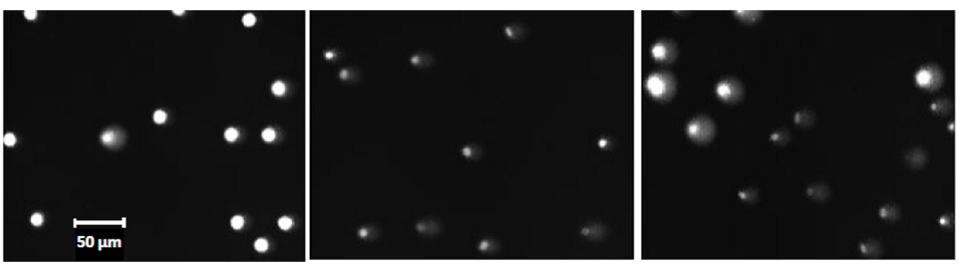

FIGURE 2 | Micrographs of control (unirradiated) erythrocytes from the turbot fish and human blood mononuclear cells. Left: human lymphocytes; center: fish erythrocytes (FE); right:1:1 mixture of the two cell types. Representative microscopic fields are shown. Size bar: 50 µm for all micrographs. See Materials and Methods for further details.

dose for both fish and human cells, except at the highest level of damage (TD > 80%) induced by 15 Gy which is beyond the dynamic range of the assay. In contrast to the DNA damage, the mean TFI does not change much with radiation dose. The slopes of the two dose-response curves are different, i.e., the comet assay indicates that less DNA damage is induced per unit radiation dose in fish than in human cells. This may be related to the lower DNA content of turbot cells, implying a smaller target size for ionizing radiation.

DISCUSSION

With the testicular cell suspensions, the comets giving strongest fluorescence represent 4c cells (i.e., premeiotic spermatocytes). This is a relatively small population (17.2% of total), compared to the larger proportion of 59.4% 1n cells (post-meiotic haploid spermatids) and 23.5% 2n (2C secondary spermatocytes plus 2n Leydig/Sertoli somatic cells); these data are all derived from **Figure 1**. In a normal experiment, the standard deviation of the small population (low numbers) of 4c cells is higher than for the other populations. We have in some cases selectively scored strongly fluorescing cells, in order to measure DNA damage in this class of 4c cells with higher precision. With automated imaging (e.g., IMSTAR^{TM} Pathfinder, Paris, France), larger numbers of cells may be scored. To estimate the ratio of 2C spermatocytes vs. 2n somatic cells, flow cytometric analysis of vimentin-stained cells may be used.

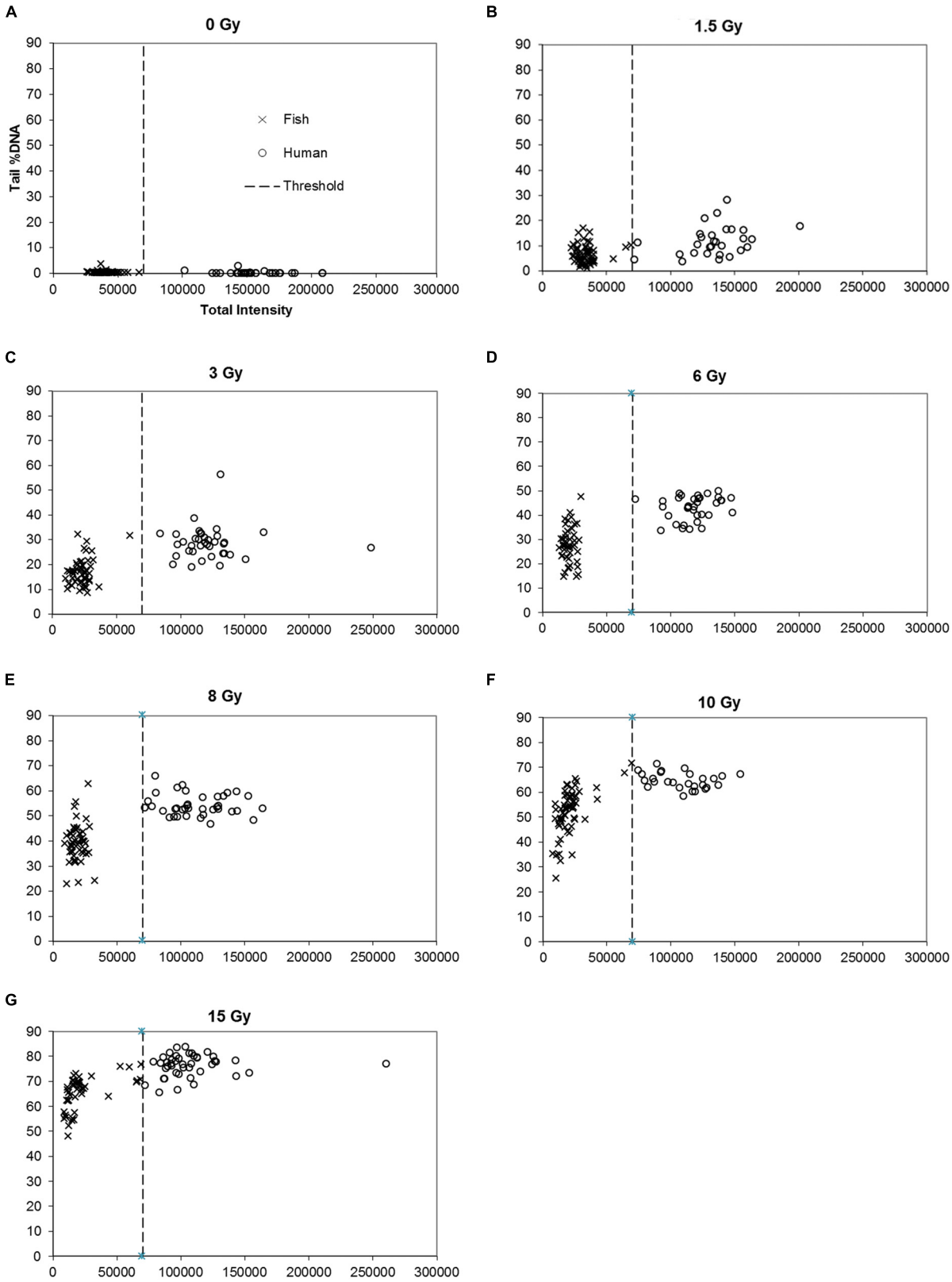

FIGURE 3 | (A–G) Mixtures of fish erythrocytes and human PBMN cells exposed to ionizing radiation. Scatter plots are shown for DNA damage (Tail %DNA, y-axis) vs. Total Fluorescence Intensity (arbitrary units, x-axis). X-irradiation doses are indicated; x- and y-axis and symbols are equal for **A–G**.

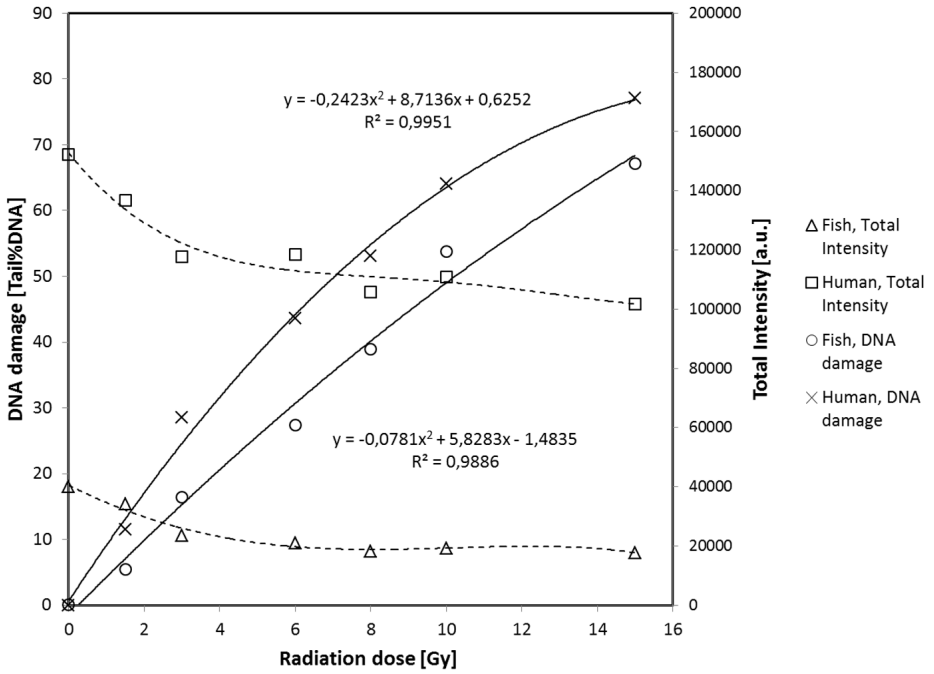

FIGURE 4 | Fish erythrocytes and human PBMN cells exposed to ionizing radiation. Left y-axis, medium level of DNA damage (Tail %DNA) vs. X-ray dose (x-axis). Right y-axis, median Total Fluorescence Insensity (arbitrary unit) vs. X-ray dose. Data are derived from **Figure 3**.

Fish erythrocytes from the turbot fish have significantly lower genome size than human cells, but there is sufficient DNA in the fish cell to produce a good fluorescent signal (**Figure 2**). Unlike the polar cod, fresh samples of the turbot showed very low background levels of DNA damage. However, turbot FE that had been frozen and stored for a few weeks at −80°C before thawing and analysis, expressed significantly elevated levels of DNA damage which also increased with longer periods of storage (data not shown). Further optimization should be performed to improve the integrity of turbot cell DNA (either FE or other cell types) upon storage. It is well known that human lymphocytes can keep their DNA integrity during freezing and storage for months, both as control and irradiated samples. The ultimate aim would be to prepare and distribute frozen samples of fish cells, either untreated or treated with defined doses of ionizing radiation inducing known levels of DNA damage. Such cells can be used as standards for calculation of frequencies of induction of DNA lesions in sample cells treated with genotoxicants. As an alternative to frozen cells, fresh blood samples may be obtained cheaply and reproducibly from fish living in an aquarium and the same fish may be sampled many times. Collection of blood from the tail vein is not known to harm the fish or to induce disease or pathological changes. The trivial fact that the turbot is a flatfish contributes to easy handling and blood collection, even for untrained personnel. One milliliter of peripheral blood contains sufficient numbers of erythrocytes for 100s of experiments.

The fluorochrome used in these analyses does not seem to be crucial, since we obtained consistent results with both ethidium bromide and SYBRGold. However, care should be taken to avoid saturation of the light signal, since the quantitative relationship with DNA content would then be distorted.

Cells of different ploidy, or in different stages of mitosis and meiosis, may be analyzed using the methodologies described here, and this could represent a cheap (although much more time-consuming) alternative to staining and analysis of the cell cycle distribution of a cell population, or of mixed cell populations, by flow cytometry. Furthermore, very few (as low as 100) cells are needed. Kruszewski et al. (2012) recently used the same methodology to show that the fluorescence intensity corresponds to the position in the cell cycle of dividing cultures. The effect of the cell cycle on induction and repair of DNA damage may this be analyzed. In conclusion, the methods described here represent new applications of the comet assay. Some further validation should be useful.

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High throughput sample processing and automated scoring

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The comet assay is a sensitive and versatile method for assessing DNA damage in cells. In the traditional version of the assay, there are many manual steps involved and few samples can be treated in one experiment. High throughput (HT) modifications have been developed during recent years, and they are reviewed and discussed. These modifications include accelerated scoring of comets; other important elements that have been studied and adapted to HT are cultivation and manipulation of cells or tissues before and after exposure, and freezing of treated samples until comet analysis and scoring. HT methods save time and money but they are useful also for other reasons: large-scale experiments may be performed which are otherwise not practicable (e.g., analysis of many organs from exposed animals, and human biomonitoring studies), and automation gives more uniform sample treatment and less dependence on operator performance. The HT modifications now available vary largely in their versatility, capacity, complexity, and costs. The bottleneck for further increase of throughput appears to be the scoring.

Keywords: high throughput, comet assay, genotoxicity, *in vivo* sample processing, automated scoring

INTRODUCTION

The comet assay in its basic form is a sensitive and relatively simple assay requiring little instrumentation. The original method (Ostling and Johanson, 1984) was later improved and standardized by Tice and co-workers (Singh et al., 1988). It involved up to three layers of agarose on a glass slide: a support gel, to which the mixture of agarose and cells is added as a second layer, and then a cover gel. Each layer needs a glass coverslip which is then removed once the gel has set. These operations cannot easily be automated. In recent years, various simplifications and also modified protocols have been presented, e.g., reducing the number of gel layers from three to one, introducing other substrates and formats than glass microscope slides, and skipping the coverslip entirely. These revisions make the assay more amenable to automation and high throughput (HT). Other innovations relate not only to the analysis part of the comet assay, but also to those elements consisting of cell cultivation, *in vitro* exposures to genotoxicants, as well as processing and storage of samples from *in vivo* exposed animals. Various forms of HT methods have appeared, with the potential to increase the number of samples that can be treated in one experiment from a maximum of ∼40 in 1 day, to at least 1,200 (Gutzkow et al., 2013). This is useful since it saves time and money, but also since other types of experiments are possible, such as the analysis of multiple tissues from animals exposed *in vivo* and large biomonitoring studies.

A protocol for the *in vivo* comet is now close to being approved by OECD (OECD 2013; Pant et al., 2014). Furthermore, European Food Safety Authority (EFSA) has issued guidance on the minimum requirements for the *in vivo* comet assay (EFSA, 2012). However, these protocols do not include or discuss HT modifications. The EC Regulation on chemicals and their safe use, REACH, obliges the chemical industry to test chemicals produced at volumes above 100 tons per year for toxic effects on health and the environment – a task that would benefit from HT methods. Hardly any alternatives to the comet assay are available for the detection of genotoxicity in specific organs *in vivo*: mutation analysis with transgenic rodents (TGR) is costly and requires specific strains of animals (OECD, 2013). There is therefore an obvious need for a reliable and validated HT comet assay preferably with some degree of automation.

We here review the most relevant HT comet assay systems, all of which have appeared during the last 15 years, and we briefly discuss their main features. We also discuss some methodological approaches which need further evaluation and do not yet offer an increased throughput but which nevertheless seem to have a potential for HT.

COMET ASSAY MODIFICATIONS

Much emphasis has been placed on avoiding the laborious two or three layer agarose gel sandwich and the use of coverslips. Several routes have been followed: (i) Modifications of glass formats; (ii) polyester films to replace glass slides; (iii) microtitre wells used for cell growth and gel forming; and (iv) more advanced technologies including cell microarrays, and microfluidics.

MINIGELS ON GLASS SLIDES

Collins and coworkers (Shaposhnikov et al., 2010) developed a format based on minigels separated on a standard glass slide by means of a silicone gasket clamped to the slide, using a tailor-made aluminum/plastic holder (see Figure 1 in Shaposhnikov

et al., 2010). Cell-agarose samples are added to each of the 12 wells and may be subjected to different lesion-specific endonucleases or other specific treatments such as fluorescent *in situ* hybridization with different DNA-probes for staining, using the same slide. In addition, the unit has a special application in studies of DNA repair capacity of cell extracts. Most recently the 12-gel glass slide format was used in a method designed for assessing BER and NER repair capacities in frozen tissues from cancer patients and healthy controls (Slyskova et al., 2014). Most electrophoresis tanks hold 10–20 slides, and so running a few 100 samples in one experiment is well within reach.

MORE SAMPLES ON A GLASS SURFACE

The first Comet assay commercial kit was described by Lemay and Wood (1999). Areas on a glass slide treated with a proprietary technology provide immobilization of gel samples, and there are hydrophobic spacers to separate neighboring samples (Trevigen CometSlide^{TM}; Trevigen Inc., Gaithersburg, MD, USA). Samples are added manually and the glass plates are treated and electrophoresed in the same way as in the traditional assay. More recently, larger glass slides are offered from the same supplier holding 96 samples. The slides are rather expensive and add significantly to the total cost of the assay, although the smaller size of the gels reduces the consumption of chemicals. A format of $4 \times 5 = 20$ samples has been used in some studies (Reelfs et al., 2011; Yuan et al., 2012; Jackson et al., 2013), but the glass formats (at least the $\times$96 version) do not seem to have been subjected to a systematic validation vs. the standard method. Jackson et al. (2013) recently successfully adapted the $\times$20 Trevigen glass slides to automated scoring by means of IMSTAR^{TM} Pathfinder.

Ritter and Knebel (2009) described a system involving 20 samples spotted onto glass slides (prototype comet slide, patent pending). Scoring was with an automated system developed by the authors; its principles and function were described in some detail. No further information was provided on the software and potential availability for other comet users. The authors describe the high reproducibility of the system and it is argued that it is suitable for higher throughput genotoxicity testing.

An increase in the number of samples per glass slide has also been described by Zhang et al. (2011) who used a plastic device to spread out five or more 20 μL samples on each standard glass slide (with no coverslips). The method was used to study effects of one chemical (melatonin) on the repair of DNA damage induced by UV-B in *Gentiana* protoplasts. The authors claim that the sensitivity is retained compared to the conventional assay and that it is easy to use. However, no further validation was reported.

POLYESTER FILMS REPLACE GLASS

McNamee et al. (2000) were the first to describe how comet assay agarose gels could be attached to a coated polyester film, thus replacing the glass slide. The GelBond® film is a thin unbreakable film used as a support for agarose gels in general. Twelve square gels are molded per film by means of plastic frames (SuperCell chambers), with no coverslips, and four films may be electrophoresed together in one tank. The method was validated using hydrogen peroxide and ionizing radiation, and the results for sensitivity were similar to those reported for the traditional assay. After scoring, the dried films may be stored securely, requiring little space. After its first publication, the method has been used in a number of laboratories. We subsequently replaced the disposable SuperCell chambers with a brass plate with cylindrical openings lined with Teflon, allowing 12 round samples (each of 30–70 μL) to be added to one GelBond® film of size 70 $\times$90 mm (Hertel-Aas et al., 2011). However, the number of samples was still rather modest (48 samples per electrophoresis).

We recently took the polyester film technology further, to accommodate up to 96 minigels on one Gelbond® film in a 96-well format, but with no use of molds, wells, or separating surfaces (Gutzkow et al., 2013). This was possible, since – with a small volume of gel (3–6 uL) – a droplet added to the cold film surface forms a uniform lens-shaped disc (see Figure 2 in Gutzkow et al., 2013). The agarose/cell samples are applied with a multipipette; a template is used to position the center of each sample. Such samples (minigels) settle within seconds on a cold surface. [Ostling and Johanson (1984) termed their technique *a microelectrophoretic study*, whereas Tice and Singh (Singh et al., 1988) used the expression *microgels*; our gel samples are of microliter size and indeed much smaller, but we use the term minigels since they are not of the micro scale which is now often used in molecular gel electrophoresis.] The film, previously cut to the size of a standard microtiter plate format, is at all stages of the comet assay attached to a plastic frame for ease of manipulation and to protect the gels (see Figure 2 in Gutzkow et al., 2013). We have processed 1200 samples in parallel in three electrophoresis tanks each holding four films. Processing (per sample) takes in total (but excluding scoring) 5–10 times less time than with glass slides (Gutzkow et al., 2013). The system has been validated using ionizing radiation to induce defined numbers of DNA strand breaks per cell, and it was verified that the 96-minigel format has the same sensitivity and dynamic range for detecting DNA damage as the standard assay based on glass slides (see also McNamee et al., 2000). For detection of base damage, parallel films are immersed in appropriate DNA repair endonuclease solutions, such as formamidopyrimidine DNA glycosylase for oxidized purines, or denV (T4 endonuclease V) for UV-induced damage. A silicone gasket and a bottomless microtiter well plate can be used to treat individual samples with chemicals or enzymes in much the same way as with the glass minigel system (Shaposhnikov et al., 2010). Scoring is done either with a semi-automated system (Comet Assay IV, Perceptive Instruments, Bury St. Edmunds, Suffolk, UK), or with the fully automated system of Imstar Pathfinder^{TM} MLA (Paris, France). This is a simple, versatile, and low-cost HT format, which we have used with a variety of cell types and tissues. Of particular importance is that the samples never fall off the film surface, even after extended lysis times (weeks) which are sometimes needed for logistic reasons (for example when preparing cell samples from fish in the open sea, for subsequent comet assay analysis; personal communication, Professor Ketil Hylland, University of Oslo). Robotic application of samples can be used to achieve precise positions of samples facilitating automated scoring. The minigel system is amenable to full automation of all steps, including addition of samples and processing of films.

ADVANCED METHODOLOGIES

Several more advanced formats have appeared in recent years. Stang and Witte (2009) developed a special 96-well multi-chamber with an agarose-containing bottom plate to which cells are attached; they may be cultured and also exposed in these wells. The multi-chamber integrates a viability assay which gives valuable information on cell status prior to comet assay. After cell treatment, the bottom plate is detached from the chamber structure and undergoes standard comet assay analysis. Originally developed for adherent cells, there is no need for detaching and harvesting the cells and the comet assay can be run immediately after cell exposure. Depending on cell type, the cells need up to 16 h to attach to the multi cell-chamber plate prior to exposure. The technology was later adapted to non-adherent cells including lymphocytes (Stang and Witte, 2010) and the authors combined this system with fully automated scoring of comets (Stang et al., 2010) using MetaSystems CometImager, thereby decreasing the evaluation time for comets by a factor of 10. In 1 day 400 samples could be fully processed. Validation was performed using methyl methanesulfonate (MMS) and H_2O_2 treatment of cells, and results were compared with those from semiautomated systems. However, the maximum level of DNA damage used in this evaluation was relatively low (Tail% DNA not above 45%). It is our experience that automated scoring systems may be less able to accurately identify and measure heavily damaged cells, resulting in reduced sensitivity and dynamic range. Nevertheless, the system of Stang and Witte (2010) represents a substantially increased throughput, integrating cell exposure, the comet assay, and also the scoring. Although several manual operations seem to be involved, the system deserves to be named HT. We have however, not been able to identify publications from independent laboratories using these methods after their first publication.

Engelward and co-workers described a very interesting method in which single cells are trapped in an array of agarose on a GelBond® film (Wood et al., 2010). Microwells of size 19–54 μm are produced using a microfabricated stamp; cells are added and are captured in these microwells by gravity (taking 0.5–1 h), at defined numbers (1–10 cells, depending on well size). The untrapped cells are aspirated and washed off before agarose is added to fill the microwells. These arrays may be fixed to a bottomless microtiter plate, allowing specific chemical treatment of cells in each of 96 wells, either before or after adding agarose. After lysis, different enzymes or repair inhibitors can be applied to nucleoids in microwells to measure different types of DNA lesions and their repair. The technology reduces the problem of overlapping comets. Furthermore, cells are trapped in one focal plane which facilitates cell location and automated scoring. The concept, which is named CometChip, has several advantages; for example, cell aggregates were efficiently analyzed for DNA damage (Wood et al., 2010). The CometChip works both with non-adherent and adherent cells. The inventors argue that the array can be mass-produced and that the assay is simple, however, the CometChip appears to be somewhat more technically demanding than the more traditional methods, and the application of cells is also more time-consuming. Some validation of the CometChip has been reported (Weingeist et al., 2013) and it was recently used by the same authors to analyze the genotoxicity of five types of nanoparticles (Watson et al., 2014).

An *in situ* comet assay substrate was developed by Mercey et al. (2010). A three-dimensional agarose layer was covalently bound to a glass slide and micropatterned into structures of defined sizes. Polarized cells keep their polarity and their differentiated state in these structures. A micropattern of 900 μm × 900 μm was used to analyze the genotoxicity of MMS, followed by a standard comet assay analysis. Scoring took place with confocal microscopy. The results obtained with this method indicate that it is suitable for HT cytotoxicity and genotoxicity screening, with automated scoring. This is an interesting method with potential for HT testing, but it is technically demanding and probably has a long way to go to become a generally usable comet assay. The operations appear to be technologically more challenging than the other formats for HT comet analysis, and there seems to be no follow-up since the method was first presented in 2010.

Li et al. (2013) recently described a novel concept based on a microfluidic chip. A 100 channels in agarose, each of height and width 20 μm×20 μm, length 20 mm, are positioned on a single glass slide. Cells (10,000) are introduced in these channels and subjected to comet analyses more or less as in the conventional assay. Electrophoresis takes place perpendicular to the channels. Since the cells are positioned precisely along the channels, their comet tails can be analyzed efficiently. This fascinating approach has great potentials but has so far been neither validated nor developed into a standardized comet assay.

CELL TREATMENTS AND SAMPLE PROCESSING

Efficient treatment of cell samples is an essential part of a HT assay, whether the cells are cultivated and treated *in vitro* or are derived from *in vivo* experiments. Considerable efforts have been made to design satisfactory logistics for the comet assay: The assay itself should be able to analyze large numbers of samples, but this has no value if high-quality samples cannot be processed in sufficient numbers for the subsequent HT comet analysis. For *in vitro* exposures, this problem may be overcome in different ways. For instance, Kiskinis et al. (2002) described an integrated exposure assay in which cells in one 96-well plate are treated with several test chemicals per experiment. Also cytotoxicity tests are performed in the wells, but cell samples are thereafter taken out and analyzed with a standard comet assay. This is clearly not a HT system, but the approach increased the genotoxicity testing throughput by more than twofold.

The HT comet assay is a must for analysis of multiple samples collected in large biomonitoring studies, prospective cohort studies, clinical trials, or in large-scale toxicology screening tests. All these study types involve many samples, often collected over long periods of time, sometimes at several locations. For logistical reasons, it is not always possible to analyze the samples when they are fresh, and freezing the samples is therefore an alternative. Although freezing of samples has been criticized (Azqueta and Collins, 2013), there is accumulating evidence that many tissue samples may indeed be snap-frozen and stored without compromising DNA integrity (Pant et al., 2014). Rigorous control of methods is needed to avoid introduction of spurious DNA damage during post-exposure processing. An optimized protocol

for freezing and thawing cells and tissues was recently described (Jackson et al., 2013), suitable for large animal experiments. Both the freezing and the thawing may be critical for preservation of DNA integrity. Cells/tissues can be frozen directly as small subsamples or as cell suspensions in freezing medium with 10% DMSO (Recio et al., 2012; Jackson et al., 2013), but snap-freezing of blood cells is also possible (Al-Salmani et al., 2011; Akor-Dewu et al., 2014). Frozen cell samples have been distributed as part of inter-laboratory trials (Forchhammer et al., 2010; Ersson and Moller, 2011), and frozen human whole blood or mononuclear cells are used as markers of environmental or dietary exposure (Collins et al., 1997a, 2014). In such studies, samples should be from a single bulk collection, to avoid seasonal, and lifestyle variations, again necessitating freezing (Moller et al., 2000; Slyskova et al., 2014).

Freezing multiple tissues from animal experiments should be well received by society since this often represents less use of animals (Pant et al., 2014). The cosmetic industry is not allowed to use animal testing and is in the process of developing human reconstructed skin models, and such 3-D systems are now under validation for both the micronucleus and the comet assays. The reconstructed skin comet project (part of the 3 D-skin project set up by the Cosmetics Europe Genotoxicity Task Force), based on EpiDerm^{TM} tissues, has completed the first validations (phase 1 and 2), and claims good intra- and inter-laboratory reproducibility. Several chemicals were tested with apparently good reproducibility, but the last step (phase 3) in this validation met with some challenges such as inter- and intra-laboratory variability and high background of solvent controls due to an insufficient quality of the tissue (Pfuhler et al., 2014). A need for optimizing and standardizing the protocol for tissue preparations is clearly indicated.

SCORING OF COMETS

The need for efficient scoring methods increases dramatically with the HT systems described above. Semi-automated scoring is highly time-consuming and easily becomes the bottleneck: an average 96-spot comet assay scoring, with 30–50 comets per samples, takes at least a day to perform. Software-based methods for unattended comet scoring are now available. They increase the efficiency at least 10-fold and they avoid tiring microscope operations.

The automated systems which are available in principle perform scoring in the same way as the semi-automated scoring systems (Perceptive Instruments Comet IV; Kinet Imaging, Andor; and others) but they to the job more quickly and with little or no operator interaction. A comet is identified and focused, the image is stored, and the system performs the image analysis to determine comet tail parameters. Two commercial systems are known to us: MetaSystems CometImager, and Imstar Pathfinder^{TM}. The performance of these systems has been described in some detail (Stang et al., 2010; Azqueta et al., 2011; Sharma et al., 2012). However, the speed, sensitivity, dynamic range, and need for operator intervention are still important issues. In particular, faint comets represent a challenge, since their head and tail lengths are difficult to measure. The commercial systems were originally developed for scoring comets in one or two samples on glass slides, and they have an automated slide feeder as an option. In recent years both systems have been adapted also to other formats, namely multiple samples on glass or polyester films.

The MetaSystems CometImager has been around for many years and has been used also to score comets in samples on GelBond® films of the same size as glass slides (personal communication, Dr. G. Koppen, VITO, Belgium). The system presents a gallery of images after scoring, which the operator may scan through quickly to delete atypical comets and artifacts. However, this may introduce a potential for bias.

The authors have participated in the adaptation of the IMSTAR system to 96 minigels on GelBond® films and also 20-well Trevigen glass slides. We score our format of 96 rehydrated/stained minigels (Gutzkow et al., 2013) on films which are either wet (i.e., with a large coverslip covering the total film surface) or semi-dry (i.e., dried for a few hours or days). Some problems in the past in identifying the faint comets (high levels of DNA damage) now seem to be solved. There is little or no difference in the slope of X-ray dose-response curves obtained with the automated (IMSTAR) vs. the manual (Perceptive) system, and the dynamic range is the same. It takes 2–4 h to analyze 96 minigels, implying that a large experiment (four films, 400 samples) can be scored in 1–2 days. Manual scoring would have taken 4–8 days. The Trevigen slides are stained according to the manufacturer's protocol, i.e., dried samples are rehydrated and stained, using antifade and coverslip (antifade and coverslips may be omitted; A. K. Sharma (Technical University of Denmark, Søborg; personal communication). Eight slides (160 samples) can be scored in a day vs. semi-automated scoring which may take 8 days depending on the number of comets scored per sample.

Automated systems are superior not only in speed but also in avoiding operator-dependent bias. For example, operators tend to select round and undamaged comets in a background of heavily damaged and overlapping comets. In any case, overlapping comets cannot be scored by image analysis, whether automated or not [with a possible exception described in (Wood et al., 2010) for cell aggregates]. This problem may be solved in an automated system by always requiring a certain space next to the comet whether there is a tail there or not. Cell density is critically important in the HT versions. The minigel system should ideally have ∼400 cells per 4–μL minigel. Making parallel samples with different cell numbers is a good option. Trevigen slides should ideally have ∼1000 cells per 30 μL well.

CONCLUSION AND PERSPECTIVES

Some of the HT systems described here rely on cutting edge technology, whereas others represent minor and low-cost modifications of the original assay. The latter ones are already available commercially or can be introduced into a normal laboratory with little or no need for special equipment. (The authors may be contacted concerning the 12 minigels on glass slides and the 96 minigels on GelBond® film.) It is expected that microwell and fluidic technology will be introduced in future versions of the comet assay. The cost of such systems may be a limitation to their use. Concerning comet scoring, we anticipate that new principles for quantitative determination of the tail magnitude will appear, possibly based on specific staining of single- and double-stranded DNA (Collins et al., 1997b).

The approved protocol for the *in vivo* comet assay which will soon be published by OECD (2013), is based on the traditional comet assay system and does not discuss HT modifications. A consequence of this is that any new version of the comet assay should be validated, at least if intended for use in genotoxicity testing and regulatory toxicology.

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On the search for an intelligible comet assay descriptor

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The comet assay has developed over the past 30 years and today, a variety of different DNA lesions and DNA repair can be measured by different versions of the assay (Collins, 2004). In the final step of the method, an image resembling a comet with a head (the nuclear core) and a tail (consisting of mainly single stranded DNA that has migrated out from the cell nuclei) is analyzed. The magnitude of the comet's DNA-tail provides information about the level of DNA lesions in the cell. The results from comet assay analyses are reported using different descriptors, the most frequently used being percentage of DNA in the tail (%T), tail length and tail moment (the product of %T and tail length). These descriptors can be reported in different ways, i.e., as means, medians or as distribution patterns. To compile the information on the migration of thousands of comets into a single value that is meaningful to convey to other researchers, is difficult. The solution has been practical and controlled by those researchers with the longest experience with the comet assay. In this opinion paper, we revisit the search for a commonly accepted descriptor for DNA damage measured by the comet assay. We define the "best" comet assay descriptor as a measurement that best describes the migration of DNA in each comet in the agarose, fits the distribution of comets in the gel, and conveys the technical measurement of comets as a descriptor that other researchers can understand. It should be emphasized that we do not embark on a mission to promote only one comet assay descriptor.

WHAT IS THE BEST DESCRIPTOR OF THE DNA MIGRATION IN THE AGAROSE GEL?

Figure 1 outlines the number of comet assay publications and certain events in the development of comet quantification. The analysis of the comets (the final step of the assay) has progressed from the initial measurements of DNA migration (length) with an eyepiece micrometer, through semi-automatic image analysis of digitized comet images by software programs, to fully automatic systems with integrated tracking and image analysis of comets (Azqueta et al., 2013; Jackson et al., 2013). This equipment offers new possibilities to analyse comets in ways that were not previously possible. In addition, the fully (or semi-) automatic image analysis systems probably lift some of the restraints in the assay that are related to the manual measurement of each comet in the gels.

The majority of publications describing comet assay results adhere to the assumption that reliable information on the DNA migration in comets can be obtained by measuring %T. At an early stage, it was suggested that the tail moment gave a better description of the DNA migration than the more simple measurement of tail length or %T (Olive et al., 1990). An objection against tail moment has been that it is difficult to visualize the comets based on this descriptor. More refined ways of describing the DNA migration in the comets (e.g., "tail inertia" or "tail profile") have not caught on Hellman et al. (1995); Bowden et al. (2003). This might have been due to the debate about tail moment or to the fact that these descriptors were not part of the software package for comet analysis at that time.

An alternative to the image analysis systems is the visual scoring system. This is based on a simple classification of the comets into (most commonly) five different classes, depending on the appearance of the comet (Gedik et al., 1992). This way to classify comets has been shown to be reproducible between laboratories scoring the same set of slides (Garcia et al., 2004). Although it is perceived as being less quantitative than computer-based image analysis systems, there are to the best of our knowledge no studies that have actually compared image analysis to visual scoring system in a systematic manner across laboratories.

WHAT IS THE BEST DESCRIPTION OF THE DISTRIBUTION OF COMETS IN A SAMPLE?

Most laboratories measure the DNA migration by software systems in 50 or more randomly selected comets per gel in a minimum of 2 gels (Tice et al., 2000). This consensus is based on both practical and statistical considerations. For the visual classification system it has been common practice to score 100 comets per gel, which is probably because it is a

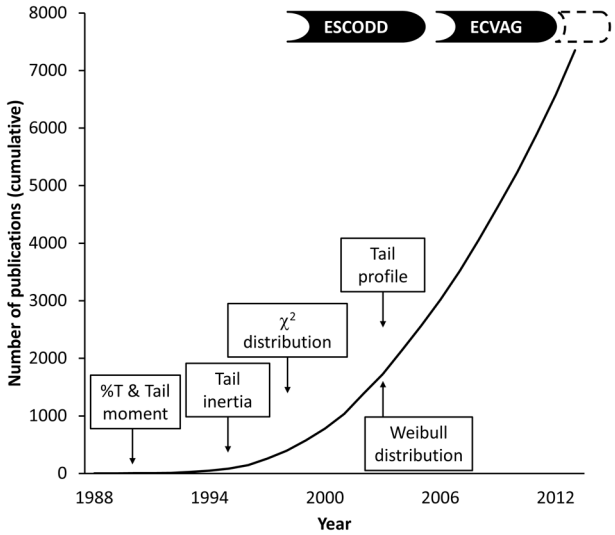

FIGURE 1 | Number of publications in PubMed using "comet assay" or "microgel electrophoresis" as search term and introduction of comet descriptors (see text for references).

relatively fast way of measuring the DNA migration and therefore one can afford the luxury of scoring more comets. However, it has been shown that increasing the number of scored comets per sample is associated with lower inter-sample variation and thereby with increased statistical power (Forchhammer et al., 2008; Sharma et al., 2012). These analyzed comets are not independent since they originate from the same sample (derived from a single experiment or measurement point). It is therefore common practice to regard the mean or median score of the comets originating from one sample as a single value. The damage (i.e., DNA migration) levels in the analyzed comets are mostly not normally distributed. Therefore, some researchers prefer to report the data as median rather than the mean. In our experience it makes little difference in the statistical analyses whether the underlying distribution of the comets has been described by the median or mean. In fact, it can be argued that both the median and mean are rather simple ways of describing the distribution. It has been shown that the underlying distribution of the comets can be described by a χ^2-distribution (Bauer et al., 1998). The shape of the distribution is described as *number of degrees of freedom* and it is useful for the description of results that are subject to random variation. This is meaningful for the analysis of comet assay descriptors since there are heterogeneities within the gel, where comets with presumably the same level of DNA damage look different at certain positions of the gel. Nevertheless, this way of describing the underlying distribution of the comets has not been explored in detail, despite the fact that it provides a better fit of the data than the normal distribution. It has also been described that the underlying distribution can be fitted to a Weibull distribution, determined by two different descriptors, i.e., shape and scale (Ejchart and Sadlej-Sosnowska, 2003). This distribution has not been used in regular comet assay analyses, which is probably explained by the complexity of having to describe the level of DNA migration by two different values.

An often-raised question is whether the comet assay results can be analyzed by parametric tests when the underlying distribution is not normally distributed. Here it is important to keep in mind that statistical analysis is based on a descriptor for each sample (with its underlying distribution, e.g., the %T). The distribution of this descriptor score expressed as %T in e.g., peripheral blood mononuclear cells (PBMCs) from a group of humans, might be normally distributed or the data can be transformed to follow a normal distribution by for example log-transformation.

WHAT IS THE BEST COMET ASSAY RESULT TO REPORT TO OTHER RESEARCHERS?

There has been substantial debate over the years about which primary comet assay descriptor is the most relevant to use. Tail length has been discarded by many researchers since the maximal DNA migration is typically reached at low doses of exposure to DNA strand breaking agents (at least when analyzed with commonly used comet assay protocols). The debate about the use of %T or tail moment has diverted attention from the real issue of whether any of these descriptors are meaningful to researchers who are not familiar with the comet assay. These descriptors are quite seriously dependent on assay conditions (Azqueta et al., 2011; Ersson and Möller, 2011), and it would be more relevant to report DNA damage values after adjustment for the assay-specific conditions, typically by reference to standard curves. Nevertheless, reference values for DNA damage in terms of %T in PBMCs have been useful in human biomonitoring studies, which could be explained by the fact that most comet assay researchers in this specific field use similar assay conditions (Møller, 2006).

WHAT ABOUT THE USE OF A REFERENCE STANDARD?

As yet there is not a true standard in the comet assay like those that are used in chemical analyses. The use of reference standards has not yet been fully implemented, but it is recommended in published guidelines to use both positive and negative controls. There is no consensus about which agents should be used and an appropriate choice depends on the types of DNA-lesions that are measured. For instance, the detection of oxidatively damaged DNA requires a specific positive control for this endpoint. An advantage of ionizing radiation as positive control is that it can be applied both as positive control and calibration curve standard, since it is well-established how many DNA breaks a certain dose of ionizing radiation causes. The drawback is that it requires special equipment for the exposure.

The European Standards Committee on Oxidative DNA Damage (ESCODD) performed the first inter-laboratory trial to attempt a standardization of comet

assay on human PBMCs. This project focused on oxidatively damaged DNA that can be measured by the comet assay as formamidopyrimidine DNA glycosylase (FPG)-sensitive sites. It was shown that the standardized results (lesions/10^6 dG of FPG sensitive sites) were similar to results obtained with other techniques (i.e., the alkaline unwinding and alkaline elution assays) (ESCODD et al., 2005). The European Comet Assay Validation Group (ECVAG) subsequently looked further into approaches to reduce inter-laboratory variation in DNA damage by the use of calibration samples for standardization of comet assay descriptors (Møller et al., 2010). ECVAG settled on describing the DNA damage as lesions/10^6 bp rather than lesions/10^6 dG because the comet assay can be modified to measure various types of nucleobase lesions.

The first ECVAG trial assessed variation in the level of DNA strand breaks in coded cryopreserved calibration standards and test samples that had been distributed to 12 laboratories. This showed that all laboratories detected a dose-response relationship in coded samples, although there were differences in the reported values. The inter-laboratory coefficient of variation was 47% when the levels of DNA strand breaks were measured as %T or comet score, whereas it was 28% after transformation to lesions/10^6 bp via the calibration curve (Forchhammer et al., 2010). The same analysis for FPG-sensitive sites showed that the participating laboratories could detect a dose-response relationship in coded cell samples. The conversion of %T to lesions/10^6 bp increased the percentage of total variation explained by the inter-sample/subject variation from 49 to 73% (Johansson et al., 2010). A subsequent ECVAG trial looked into a standard comet assay protocol, but was only partly successful because some laboratories observed no difference in calibration curve samples and obtained negative values of FPG sensitive sites in human PBMCs (Forchhammer et al., 2012). ECVAG also showed that the overall variation of FPG-sensitive sites in the PBMCs could be partitioned into inter-laboratory (56.7%), residual (42.9%), intra-laboratory (0.2%) and inter-subject (0.3%) variation (Ersson et al., 2013). The most important finding in this trial was that the variation within each laboratory was relatively low.

Variation in DNA damage can be diminished by standardization of the primary comet assay descriptor using calibration samples. As highlighted by ComNet—a network of researchers using the comet assay in human biomonitoring studies—one of the challenges is to determine experimental factors that influence reliability and robustness of the comet assay as a biomonitoring tool (Collins et al., 2014). For, these kinds of studies, it is important to have low assay variability among laboratories. The number of scored comets could be an important determinant in this respect. But, maybe we also have to look more ahead and think of developing comet assay equipment with integrated calibration samples for standardization, and/or completely other scoring principles. Still some work to be done in the next 30 years!

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Statistical analysis of comet assay results

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The comet assay can distinguish small differences in DNA damage between different samples of cells, implying that statistical tests are important to assess whether this occurs by chance. Excellent scholarly papers with concise descriptions of statistical analysis and recommendations for tests have been published (Lovell et al., 1999; Lovell and Omori, 2008). We often come across publications that unfortunately have not taken advantage of statistical models in design and analysis of comet assay results. The present commentary is based on the notion that statistical analysis of comet assay data should not be complicated, but consideration of statistical analysis before carrying out the experiments typically makes it much easier to analyse the results.

WHY DO WE PERFORM STATISTICAL ANALYSIS OF COMET ASSAY RESULTS?

Statistics are typically done to prove that the DNA damage levels are different between groups, although we formally test for no difference between groups. By default the probability of rejecting the null hypothesis is 5%, although this value is not sacrosanct. Nevertheless, *P*-values less than 5% can make the difference between publishing in prestigious journals or not. Therefore, there is a certain impetus toward producing low *P*-values and misconception of what it really means.

MISCONCEPTION 1

The *P*-value (e.g., $P < 0.05$) does not indicate that probability of the null hypothesis being true (i.e., $P(H_0|R) < 5\%$). On the contrary, the *P*-value is the probability of the observed result given the null hypothesis is true (i.e., $P(R|H_0) < 5\%$). It means that if we did the experiments again, there would be less than 5% chance that the DNA damage level was the same between groups.

MISCONCEPTION 2

The *P*-value does not describe the magnitude of biological effects, because it depends on the variation of DNA damage and number of observations. Datasets with little standard deviation and large number of observations can be highly significant in statistical analysis.

MISCONCEPTION 3

The *P*-value does not indicate strength of the association between exposure and DNA damage because it depends on the experimental design. For instance, *P*-values from experimental designs with multiple groups or interactions are much more convincing than simple designs with only two groups. In addition, the *P*-value from parametric tests tends to be more convincing than non-parametric tests.

WHAT IS THE EXPERIMENTAL UNIT?

In a traditional comet assay study the investigator measures DNA migration in a number of *Comets* from each *Sample* (e.g., blood sample or tissue from one individual). *Samples* in cell culture experiments refer to independent experiments on different days, preferably with cells from different passage number or donors. It is common practice to measure DNA migration in at least 50 *Comets* per *Gel*. There are often two replicate *Gels* per experiment (i.e., one day of analysis). Consequently, there are usually 100 measurements of DNA migration per *Sample*. This is described as a hierarchical nested experimental design where *Comets* are nested within *Gels*, *Gels* are nested within *Samples*, and *Samples* are nested within *Treatment*. However, it is very important to acknowledge that *Comets* in the same gel have been subjected to the same assay procedure and they are therefore not independent observations. Inclusion of all *Comets* in the statistical analysis is therefore a severe violation of the principle assumption that the statistical analysis is based on independent observations. When evaluating *in vivo* data, the animal is the experimental unit.

The issue about the experimental unit was already discussed extensively in the 1990s and it was clearly stated that "the sample rather than the cell is the experimental unit" (Lovell et al., 1999). Nevertheless, it appears that certain investigators integrate individual *Comets* in the statistical analysis (Bright et al., 2011). Unfortunately, it appears that commercial suppliers also use individual *Comet* data in their instruction for comet assay analysis (e.g., Trevigen Instructions, Catalog #4256-010-CC).

WHAT IS A STATISTICAL ANALYSIS?

The statistical analysis basically compares the variation between known variables (e.g., exposure groups) with residual variation (e.g., assay variation). However, we rarely know the residual variation and therefore assess it in the same experiment as the known variables. Therefore, it is best to have as many data in the statistical analysis as possible because it provides a better determination of the residual variation. In the statistical analysis, we first calculate the total variation, thereafter the

variation related to the known variables, and this subtracted from the total variation should give the residual variation. Because of this procedure, the variation within different groups should be similar (i.e., homogeneity of variance). In addition, the residuals (i.e., difference between the observed and expected value, based on the statistical model) should have a normal distribution because it principally is caused by random variation.

CAN PARAMETRIC TESTS BE USED FOR COMET ASSAY DATA?

The distribution of *Comets* is typically non-normal. This sometimes leads to the misconception that comet assay data cannot be analyzed by parametric tests. As an example, **Figure 1** outlines a dataset of human peripheral mononuclear blood cells that have been exposed to ionizing radiation. This statistical analysis is applicable to cell culture, animal and human results. There are 3 *Samples* for each ionizing radiation dose, each *Sample* being the data derived from measuring DNA damage in 50 *Comets*. As example of a statistical question, we want to assess the magnitude of effect generated by 5 Gy of ionizing radiation in cellular DNA damage.

Figure 1A reveals that the distribution of *Comets* is non-normal at low doses, while it seems to follow the normal distribution at high doses. **Figure 1B** shows the dose-response relationship, each symbol being the mean value of the individual *Comets*. Although there are different distributions of individual *Comets*, there is a linear relationship between the radiation dose and DNA damage level. **Figures 1C–E** display a high correlation between values that have been obtained from the mean, median or geometric mean of the individual *Comets*. Indeed, it makes little difference using the mean or median of *Comets* of even highly skewed distributions in the present dataset.

The data in **Figure 1B** can be analyzed by either parametric or non-parametric tests, depending on the homogeneity of variance and distribution of residuals (i.e., the unexplained variation). There are a range of different *post-hoc* testsparametric tests, including Dunnett's, Fisher's least statistical difference, Scheffe's and Tukey's tests. Given a hypothesis of a linear relationship between the dose and DNA damage, these data can be analyzed by regression analysis. However, we will in this example use one-way analysis of variance (ANOVA), implying no *a priori* hypothesis of a linear relationship. First we test for homogeneity of variance between the groups (e.g., by Levene's test). In this case, there is inhomogeneity of variance ($P = 0.005$). One result at 5 Gy is aberrant, which is easily demonstrated by substituting it with a dummy variable (i.e., the mean of the two other data points at 5 Gy, $P = 0.38$). Importantly, the aberrant value is higher than expected, which could be a problem because the statistical analysis may show significance due to this value only, while it does not look like an outlier. A log-transformation of the data reduces the inhomogeneity of variance ($P = 0.044$), although principally it still violates the assumption for parametric tests. One option would be to analyse the data with a non-parametric test (Kruskal-Wallis tests of ranks). This shows statistically significant ($P < 0.0156$), but a *post-hoc* Tukey-type comparison test among medians indicate that 0 and 2.5 Gy (as well as 5 and 10 Gy) are not different. Thus, a non-parametric analysis of the data is not an optimal solution and we wanted to assess the magnitude of effect. Therefore, we proceed with a parametric ANOVA, knowing the potential bias due to the aberrant value. The overall ANOVA is highly significant ($P < 0.001$). A *post-hoc* calculation of the fold-difference and 95% confidence interval (CI) shows 7.8-fold (95% CI: 7.0-8.6 fold) increased level of DNA damage at 5 Gy for data assessed on normal scale, whereas a back-transformation of the log-transformed data yields the same mean fold-difference with a slightly larger and skewed CI (6.9–8.9 fold). The CI is also larger when calculated from the standard deviation of only the three 5 Gy results (5.8–9.9 fold), although it is still highly significant as it does not include unity (unit = 1).

Overall, this example demonstrates that one can do a reliable statistical analysis on even non-optimal datasets. However, it should be emphasized that the dataset was balanced (i.e., equal number of observations in each group), whereas this may not hold true for especially datasets with uneven number of observations between groups.

WHAT TYPE OF STATISTICAL ANALYSIS SHOULD BE USED?

It should be emphasized that having chosen the statistical design before starting the experiments is a huge advantage. The type of design surely depends on the research question, but usually economic issues are important too. For instance, experiments with 4 independent variables would add up to 64 different groups in a simple full factorial design (4^4-groups). Here we describe three examples for experiments with special emphasis on the research question and study design.

EXAMPLE 1: ARE PARTICLES FROM COMBUSTION OF BIODIESEL LESS GENOTOXIC THAN CONVENTIONAL DIESEL?

To answer that question, we investigated DNA damage by particles obtained from combustion of different types of diesel in two different engines, which essentially comply with previous and present EU regulation. In addition, a reference material was included in the experiments and samples were tested in three different concentrations (Hemmingsen et al., 2011). In this design there are numerous irrelevant comparisons (e.g., high concentration of reference material against low concentration of particles from an engine complying with present EU regulation). However, we also wanted to have all data in the same model because it increases the statistical power by better determination of the residual variation. Consequently, these results were tested with nested ANOVA where concentrations were nested in particles.

EXAMPLE 2: DO DYSLIPIDEMIC MICE HAVE HIGHER AGE-DEPENDENT ACCUMULATION OF DNA DAMAGE THAN NORMAL MICE?

The question entailed a combination of linear (age) and categorical (strain) independent variables. Therefore, it was analyzed with a generalized linear model, assessing the interaction between age and strains. It showed that the two strains of mice had similar accumulation of strand breaks in the liver (single-factor effect of age), whereas there was an interaction between age and strain for oxidatively damaged DNA so that dyslipidemic mice had a higher regression coefficient as compared to wild-type mice (Folkmann et al., 2007).

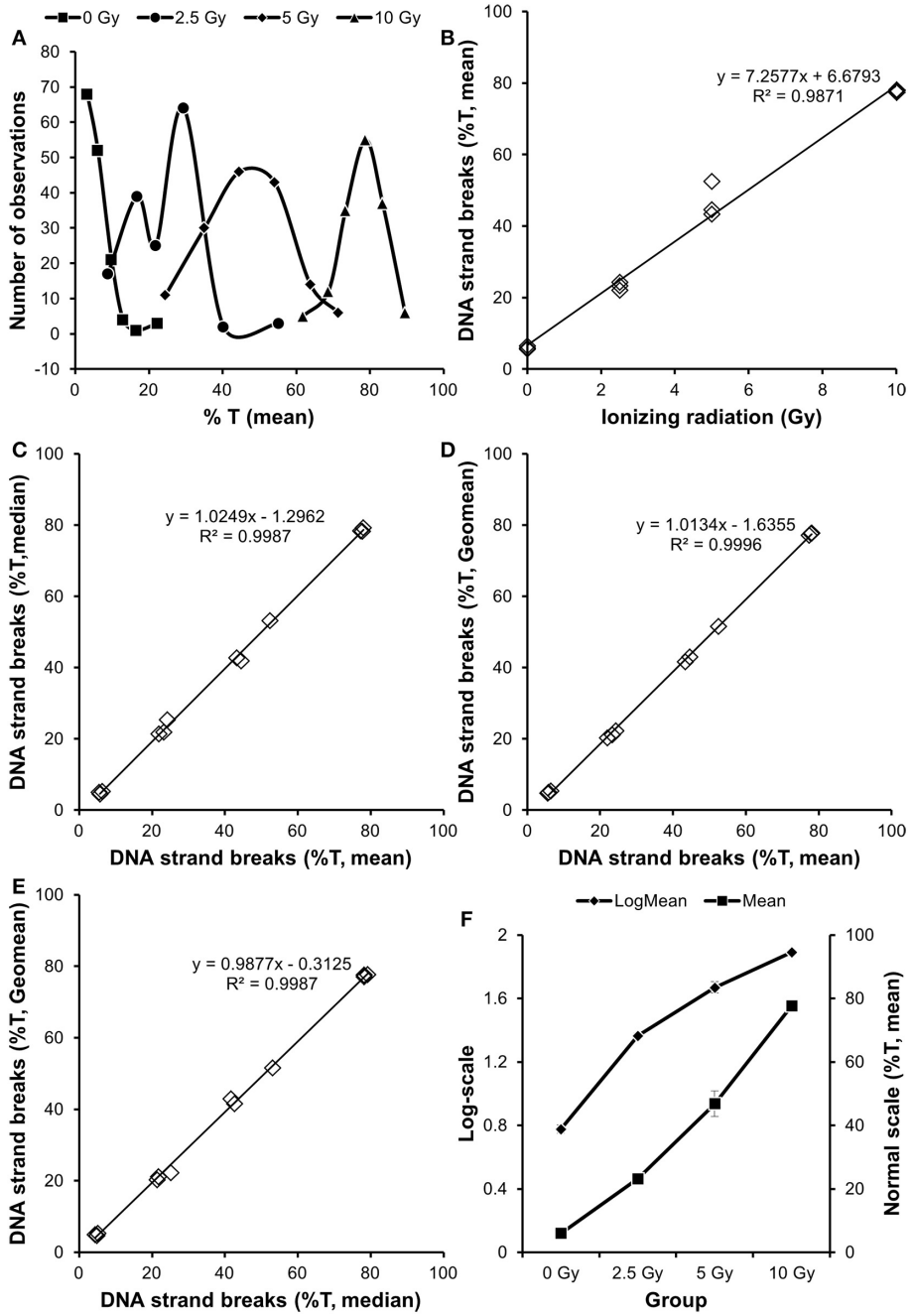

FIGURE 1 | Analysis of association between exposure to ionizing radiation and level of DNA damage. The mean values from individual *Comets* displayed variation that tended to shift dose-dependently from non-normal distribution to normal distribution **(A)**. The dataset consisted of 3 independent *Samples* per *Treatment* **(B)**. There was no difference whether the data in *Sample* was obtained from the mean, median of geometric mean of the individual *Comets* **(C–E)**. An analysis of the data by ANOVA indicated inhomogeneity of variance between groups, which was diminished by log-transformation **(F)**.

EXAMPLE 3: IS EXPOSURE TO SUNLIGHT ASSOCIATED WITH INCREASED LEVEL OF DNA DAMAGE?

The exposure to sunlight in Denmark is characterized by periods of high exposure (i.e., summer days with sunshine). This exposure was investigated in a repeated measurement study where subjects were followed for 14 months (Møller et al., 2002). Each subject was asked to give blood approximately every third week. However, the data could not be analyzed by repeated measurement ANOVA because of unequal periods of sampling for each subject and it was important to adjust for potential confounders. Therefore, these data were analyzed with a generalized linear mixed model robust to unequal

timescales, with demographic variables, nutrition, exercise, and sunlight exposure as independent variables. In addition, the DNA damage levels were assessed on fresh blood samples, together with cryopreserved control samples. The statistical analysis showed that sunlight intensity, hours spent in the sun, and sex were statistically significant variables. The remaining variation (standard deviation of residuals) was the same as the variation in the control samples, indicating that the other variables in the statistical model had no effect on the level of DNA damage.

Collectively, comet assay data can be analyzed by parametric and non-parametric tests. We recommend that the experimental design determines the type of statistical analysis and balanced designs are more robust to datasets with inhomogeneity of variance between groups or non-normal distribution of residuals.

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