Universidad de Navarra

Facultad de Farmacia y Nutrición

Optimization of the *in vitro* comet assay as a tool for mechanistic risk assessment

Damián Muruzábal Gambarte

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Memoria presentada por **D. Damián Muruzábal Gambarte** para aspirar al grado de Doctor por la Universidad de Navarra en el Programa de Doctorado en Alimentación, Fisiología y Salud. El presente trabajo ha sido realizado en el Departamento de Farmacología y Toxicología, bajo la dirección de la **Dra. Amaya Azqueta Oscoz** y de la **Dra. Ariane Vettorazzi Armental**. Considerando finalizado el trabajo, autorizan su presentación ante el Tribunal correspondiente.

En Pamplona, noviembre 2020

Dra. Amaya Azqueta Oscoz

Dra. Ariane Vettorazzi Armental

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“Ships are launching from my chest”

Welcome home, son (Radical Face)

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Abstract

Genotoxicity evaluation is of key importance in the health risk assessment of substances to which humans are exposed, as it has been long established that certain genotoxic compounds are able to damage DNA entailing severe consequences for human health, such as cancer. Current strategies of genotoxicity testing consider mainly final effects in DNA: mutations and chromosomal aberrations. However, a mechanistic approach more relevant to humans, in which not only classical endpoints but also mechanistic events (*e.g.,* DNA oxidation or alkylation) are integrated and considered for risk assessment, is becoming more necessary.

In this context, modifications to the *in vitro* comet assay protocol, which measures strand breaks and alkali labile sites in its standard version, arise as a promising alternative method in the detection of premutagenic lesions. The aim of this thesis was to develop and validate a new tool for *in vitro* genotoxicity testing, based on the comet assay, that can be used in the elucidation of different mechanisms of action. This approach may represent a good candidate for complementing current *in vitro* genotoxicity testing batteries.

The combination of the comet assay with lesion-specific enzymes is used to detect altered bases. A review about this version of the assay revealed that formamidopyrimidine DNA glycosylase (Fpg) is the most used enzyme, used for the detection of oxidized bases. In total, 12 different enzymes have been combined with the comet assay to detect other lesions such as alkylated bases, presence of uracil, pyrimidine dimers or AP-sites. The areas of application in which the enzyme-modified comet assay has been more extensively used are *in vitro* genotoxicity testing and human biomonitoring.

For the detection of alkylated bases, two non-commercially available bacterial enzymes, 3-methyladenine DNA glycosylase II (AlkA) and 3-methyladenine DNA glycosylase (AlkD), have been sporadically employed. In this thesis, a commercial human alkyladenine DNA glycosilase (hAAG) was successfully applied for the first time. Moreover, the use of hAAG together with other different enzymes (non-commercial Fpg and commercial Fpg, Endonuclease III -Endo III- and human 8-oxoguanine DNA glycosylase -hOGG1-), with various specificities towards oxidized lesions, was optimized to be used on a single assay using a medium throughput format (*i.e.,* 12 minigels/slide). To this aim, the incubation conditions when using the widely used 2 gels/slide format and the medium-throughput 12 minigels/slide format was assessed. This comparison highlighted that is crucial to perform enzyme titration experiments using the same protocol, equipment and the format that are going to be used in the final experiments.

Moreover, in order to detect DNA cross-links, an extra DNA lesion which may be difficult to detect by mean of enzymes, an already known modification of the comet assay was set up using the same throughput format.

Finally, both comet assay modifications were validated using TK-6 cells treated with non-cytotoxic concentrations of nine compounds with several mechanisms of action: oxidizing and alkylating agents, cross-linkers, a bulky-adducts inducer and non-genotoxic compounds. The combination of the results of both modifications allowed to clearly differentiate the induced lesions, with the exception of the bulky adducts, which was expected. Moreover, no DNA lesions was detected in cells treated with the non-genotoxic compounds. Both Fpg enzymes (non-commercial and commercial one) gave same results.

The *in vitro* comet assay modified with several enzymes together with the cross-links modification increases significantly the comet assay ability to detect different premutagenic lesions, providing genotoxic mechanistic information about the type of damage. Its application might be very relevant in the current regulatory context.

Contents

List of abbreviations

AlkA	3-methyladenine DNA glycosylase II
AlkD	3-methyladenine DNA glycosylase
ALS	Alkali-labile sites
AO	Adverse outcome
AOP	Adverse outcome pathway
AP	Apurinic/apyrimidinic
ATCC	American Type Culture Collection
BER	Base excision repair
BPDE	Benzo[a]pyrene diol epoxide
BSA	Bovine serum albumin
CisPt	Cisplatin
CPD	Cyclobutane pyrimidine dimer
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
ECHA	European Chemical Agency
EEMGS	European Environmental Mutagenesis and Genomics Society
EFSA	European Food Safety Authority
EMGS	Environmental Mutagenesis and Genomics Society
EMS	Ethyl mehtanesulfonate
Endo III	Endonuclease III
Endo IV	Endonuclease IV
ESCODD	European Standards Committee on Oxidative DNA Damage
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
Exo III	Exonuclease III
Fapy	Formamidopyrimidine (or ring-opened purine)
FISH	Fluorescent in situ hybridization
Fpg	Formamidopyrimidine DNA glycosylase
GC/MS	Gas chromatography/mass spectrometry
hAAG	Human alkyladenine DNA glycosylase
hOGG1	Human 8-oxoguanine DNA-glycosylase
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICL	Inter-strand cross-link
IWGT	International Workgroup on Genotoxicity testing
JaCVAM	Japanese Center for the Validation of Alternative Methods
KE	Key event
KER	Key event relationship
LMP	Low melting point
MIE	Molecular initiating event
Mit. C	Mitomycin C
MMS	Methyl methanesulfonate
NAM	New approach methodology
NER	Nucleotide excision repair
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NM	Nanomaterial
NP	Nanoparticle
NTH1	Endonuclease III-like protein 1
NTP	National Toxicology Program
ODN	Oligonucleotide
OECC	Organisation for European Economic Cooperation
OECD	Organisation for Economic Co-operation and Development
OECD TG	OECD Test Guideline
OECD TGP	OECD Test Guideline Programme
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSG	Relative suspension growth
SB	Strand break
SSB	Single strand break
T4EndoV	T4 endonuclease V
Udg	Uracil DNA glycosylase
UV	Ultraviolet
WBC	White blood cell

Chapter 1

General introduction

1. Genotoxicity, mutagenicity and cancer

Human health can be affected by the exposure to several agents, either endogenous (*e.g.,* reactive oxygen species -ROS- produced in metabolic reactions within the cell) or, more commonly, exogenous (*i.e.,* xenobiotics, such as tobacco smoke or UV light), which may be the origin of a disease or disorder. For such reasons, it is necessary to evaluate the safety of substances that may represent a risk to human health, such as pharmaceutical drugs, food and feed additives, cosmetics or industrial chemicals among others. One of the most important steps in safety evaluation is the study of the effects of substances towards DNA. Indeed, DNA is a very reactive molecule and highly susceptible to chemical modifications induced by endogenous or exogenous agents and to spontaneous processes (*e.g.,* spontaneous breakdowns or deaminations) (Lindahl and Nyberg, 1972; Shen *et al*., 1994). Therefore, processes and interactions leading to DNA damage may entail severe consequences including carcinogenic processes.

In the field of toxicology there is a specialized branch aimed to develop methods and strategies to identify compounds that can induce DNA lesions, and to analyze its mode of action to further evaluate the risks derived from its exposure. This discipline is called genetic toxicology or, more commonly, genotoxicology. Thus, in this context, any agent capable of damaging DNA molecule either directly or indirectly (*i.e.,* by interacting or damaging cellular components involved in genome fidelity regulation) is considered genotoxic. DNA damage can arise in several forms: abasic sites (either apurinic or apyrimidinic) (AP-sites), single and double strand breaks (SSB and DSB respectively), oxidized and alkylated bases, bulky adducts, intrastrand and interstrand cross-links, adducts on the phosphodiester backbone, protein-DNA cross-links and the misincorporation of a base (or analogues) (see *Figure 1*) (Azqueta and Collins, 2011).

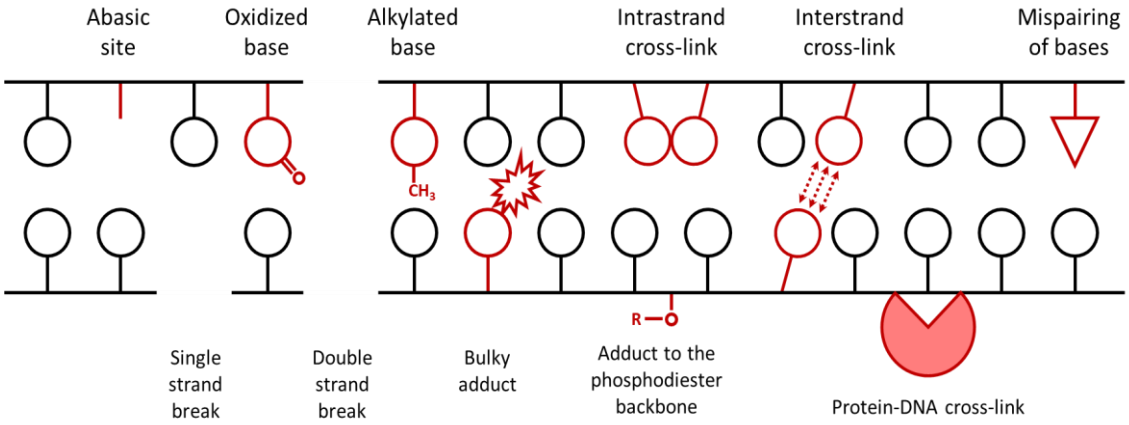

Figure 1. Schematic diagram of different DNA lesions.

The type and amount of DNA lesions determine its final effect for the cell (*e.g.,* disruptive effects in DNA transcription and/or replication and alteration in nucleotide sequence). In most cases, DNA repair machinery can repair these lesions if their levels are not too high, otherwise, apoptosis pathways are triggered (King and Cidlowski, 1995). However, sometimes DNA damage remains unrepaired (or it is misrepaired) when the cell replicates, thereby providing the basis for genomic instability and mutations (Chatterjee and Walker, 2017). For this reason, all these previously mentioned lesions are usually known as premutagenic lesions.

Mutations are permanent and detectable modifications in the DNA sequence (*i.e.,* genotype) of the cell, which is then transmitted to daughter cells and succeeding generations. Overall, mutations can be classified as gene mutations (or point mutations) when involving a single gen, or as chromosomal mutations, which affect to more genes or chromosomes. For instance, an oxidized base such as 8-oxoguanine (8-oxoGua) can produce an alteration in the base-pairing properties, since 8-oxoGua tends to pair with adenine instead of cytosine, thereby causing a point mutation (Grollman and Moriya, 1993). DSBs can potentially induce chromosomal aberrations, as they can induce chromosomal rearrangements or produce chromosomal breaks (Morgan *et al*., 1998).

Mutations and chromosomal alterations can be produced in either somatic or germ cells. Mutations that occur in somatic cells are not transmitted to the offspring, but are involved in the initiation, promotion, and progression of cancer (Basu, 2018) as well as in other non-malignant diseases (*e.g.,* Triple-A syndrome) (Jackson and Bartek, 2009). On the other hand, when these alterations occur in germ cells, they can lead to spontaneous abortions, infertility or it can be transmitted to the offspring (Jackson and Bartek, 2009).

Regarding the causation of tumors, nowadays it is possible to attribute the incidence of some of them to the exposure to particular compounds or mixtures (Whiteman and Wilson, 2016), which are commonly known as carcinogens. The initiation or promotion of a cancerous process by these chemicals, such as some metals (*e.g.,* arsenic) (Hayes, 1997), some mycotoxins (*e.g.,* aflatoxin B1) (Wogan, 1999) or tobacco smoke (Hecht, 1999), it is usually known as chemical carcinogenesis (Oliveira *et al*., 2007). Carcinogens can be divided into 2 main groups: genotoxic and non-genotoxic carcinogens (Hayashi, 1992). Most mutagenic compounds are carcinogens (*per se* or after metabolism) as they damage DNA molecule leading to mutations and initiates malignant transformations. Non-genotoxic carcinogens, on the other hand, are not mutagenic and act through mechanisms not related to DNA damage, such as cell proliferation or hormonal effects. This difference in the mechanism of action is indeed very relevant from the current

regulatory perspective. The traditional assumption is that direct genotoxic carcinogens are considered as compounds without threshold, and thus some risk may exist at any level of exposure, whereas non-genotoxic carcinogens have threshold effect, related with dose, and thus health-based guidance values can be established.

As it can be inferred, the terms of mutagenicity and genotoxicity are relatively close but are not the same. Indeed, genotoxicity is a wider concept which comprises (by definition) mutagenicity. However, this relationship is not given both ways, as not all genotoxic compounds induces mutations. Thus, measuring the levels of premutagenic lesions could be a relevant indicator or biomarker of genetic instability, although it reflects a dynamic steady state in which the damage is normally balanced by the DNA repair mechanisms. For this reason, it should be kept in mind that despite DNA damage has been identified as the initiating event in tumorigenesis, it is imprudent to consider the level of DNA damage as an indicator of cancer risk, as many other factors are involved (Poirier, 2012; Basu, 2018). Nevertheless, in human biomonitoring studies the level of DNA damage it is considered a good marker of exposure to genotoxic agents (Nikitaki *et al*., 2015).

2. Regulatory strategies for genotoxicity testing

In the first decades of the last century, observations of the effects of different types of radiation towards *Drosophila melanogaster* and different plants led to the first unequivocal evidence of chemical mutagenesis (Auerbach and Robson, 1946). Then, after the elucidation of DNA structure by Watson and Crick in 1953 (Watson and Crick, 1953) and following discoveries of DNA replication, genetic code and protein synthesis, it was possible to understand that point mutations may lead to changes in the phenotype. For this reason, many studies were focused on studying the effect of chemical compounds in DNA, revealing the harmful effects of mutagens in human health. These facts led to the foundation in 1969 of the Environmental Mutagenesis and Genomics Society (EMGS) in the USA and the European Environmental Mutagenesis and Genomics Society (EEMGS) in 1970. These societies were aimed to study and evaluate potential genetic and carcinogenic effects that the exposure to the increasing number and variety of chemicals may pose to humans. Thus, since the 1970s first guidelines with protocols of assays to evaluate the mutagenicity were prepared and new assays were developed (*e.g.,* Ames test). Indeed, the relationship of mutagenicity and carcinogenicity was also evidenced in those years, which made the necessity of including these assays in the toxicity evaluation of substances (Bello and López de Cerain, 2001).

Since then, several assays have been developed to assess the genotoxicity or mutagenicity of substances, such as pharmaceutical drugs, industrial chemicals, pesticides, environmental contaminants or food and feed additives, among others. Indeed, regulatory agencies and advisory bodies have made recommendations on genotoxicity testing strategies to assess the risk of these substances (some of these strategies are revised in the following subsections). Due to the fact that no single assay is able to measure all possible effects of compounds towards DNA, the recommended strategies for genotoxicity testing are designed as batteries of validated assays aimed to cover a spectrum of different lesions/mutations in DNA. Thus, assays are performed sequentially, first using *in vitro* experimental systems and then, if needed, *in vivo* assays are conducted.

The strategies of the assays are in line with the three Rs principle (replacement, reduction and refinement in animal experimentation), which is aimed to encourage the use of alternatives to animal testing as well as the improvement of both animal welfare and scientific quality (Russell and Burch, 1959). Since the three Rs principle was originally proposed in 1959, its adoption and implementation has been widely extended. Indeed, it has been adopted by the scientific community, regulated by different authorities (*e.g.,* European Union) (EU, 2010), and even centers for the validation of alternative methods have been established (*e.g.,* the European Union Reference Laboratory for Alternatives to Animal Testing, EURL-ECVAM) (reviewed in Hubrecht and Carter, 2019).

2.1. OECD Guidelines for the testing of Chemicals

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organization originally established in 1948 as Organisation for European Economic Cooperation (OEEC) aimed to reconstruct the continent after world war II. Encouraged by OECC success, the United States and Canada joined OECC leading to the creation of OECD in 1961 (OECD, 2020a). Nowadays, OECD goal is to promote policies by “establishing evidence-based international standards and finding solutions to a range of social, economic and environmental challenges” (OECD, 2020b).

The number of chemicals reaching the market (*e.g.,* drugs, pesticides or industrial chemicals) increases every year and all these compounds require safety testing. For this reason, one of the work areas of the OECD is the development of guidelines for the testing of chemicals to assess its potential effects on human health and the environment (OECD, 2020c). OECD guidelines for the testing of chemicals are internationally accepted as standard methods for safety testing and cover safety testing of chemicals in its broadest sense. Additionally, OECD guidelines are

regularly updated according to the current state-of-the-art in hazard characterization and are supposed to address animal welfare concerns (three Rs principles).

Regarding genotoxicity testing, guidelines for *in vitro* and *in vivo* assays are included within Section 4 (*i.e.,* Health Effects) of the OECD guidelines for the testing of chemicals. The following table (*Table 1*) compiles a list of validated assays for genotoxicity testing.

Table 1. List of OECD guidelines for different *in vitro* and *in vivo* assays for genotoxicity testing.

Guideline: Test No.	Title	DNA effect	Reference
In vitro			
471	Bacterial Reverse Mutation Test	Gene mutations	OECD, 2020d
473	*In Vitro* Mammalian Chromosomal Aberration Test	Structural chromosomal mutations	OECD, 2016a
476	*In vitro* Mammalian Cell Gene Mutation tests using the *Hprt* and *xprt* genes	Gene mutations	OECD, 2016b
487	*In Vitro* Mammalian Cell Micronucleus Test	Structural and numerical chromosomal mutations	OECD, 2016c
490	*In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	Gene mutations	OECD, 2016d
In vivo			
474	Mammalian Erythrocyte Micronucleus Test	Structural and numerical chromosomal mutations	OECD, 2016e
475	Mammalian Bone Marrow Chromosomal Aberration Test	Structural chromosomal mutations	OECD, 2016f
488	Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays	Gene mutations	OECD, 2013
489	*In vivo* Mammalian Alkaline Comet Assay	DNA strand breaks	OECD, 2016g

2.2. Genotoxicity testing approaches for the assessment of substances present in food and feed

Traditionally, pharmaceuticals have been the most widely regulated chemicals and have been subject to safety assessment evaluation for registration purposes following guidelines such as the ones from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Indeed, the ICH S2(R1) guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (ICH, 2011), is considered the gold-standard strategy for genotoxicity testing (*Figure 2*).

Genotoxic evaluation of substances is not only essential for pharmaceutical drugs, but also to other substances, such as those present (intended or not) in food and feed. In Europe, recommendations regarding genotoxicity testing strategies for food and feed are provided by the European Food Safety Authority (EFSA). This agency was founded in 2002 to be a source of scientific advice and communication on risks associated with the food chain. In this context, EFSA has published a scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011).

EFSA strategy for genotoxicity testing shares some features with ICH S2(R1) guideline (*Figure 2*). As in the first option proposed by the ICH strategy, the one proposed by EFSA considers a sequential process in which firstly two *in vitro* tests are performed (*i.e.,* bacterial reverse mutation assay and micronucleus test) to cover three genotoxic endpoints: gene mutation, structural and numerical chromosomal aberrations. Then, depending on the outcome, different decisions can be made:

- If clear negative results are obtained in all *in vitro* endpoints, no further testing is required except in some special cases (*e.g.,* when the external *in vitro* metabolic activation system does not cover the full spectrum of potential genotoxic metabolites generated *in vivo*). In general, it is considered that the substance lacks genotoxic potential.
- If positive results are obtained in one or two *in vitro* tests, *in vivo* follow-up tests are required. In this case, the *in vivo* test selected should be related to the genotoxic endpoint(s) identified in the *in vitro* tests and focused on the appropriate target organ or tissues. As seen in *figure 2*, different options are proposed. If the first *in vivo* is positive, no further test is needed, and the substance is considered an *in vivo* genotoxin. If the test is negative, it may be possible to conclude that the substance is not an *in vivo* genotoxin, although in some cases a second *in vivo* test may be necessary (*e.g.,* if there is no evidence that the agent reaches the tissue under investigation or when more than one *in vitro* test is positive).
- If equivocal, inconclusive or contradictory results are obtained in the *in vitro* tests, further *in vitro* tests are required to clarify the genotoxic potential *in vitro* (either by repeating with different conditions or by conducting a different test) and then, if needed, *in vivo* testing should be considered.

Furthermore, EFSA considers, on a case-by-case basis, variations on the recommended strategy if it is proven that the three aforementioned genotoxic endpoints are adequately investigated (EFSA, 2011).

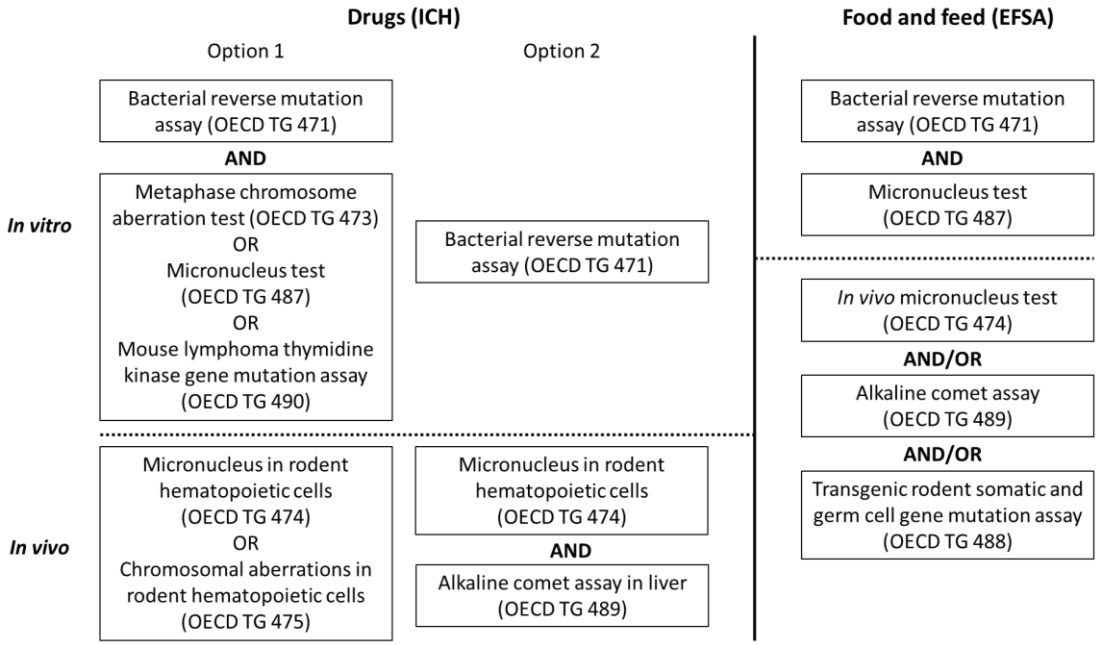

Figure 2. Schematic representation comparing the strategy for genotoxicity testing of drugs recommended by ICH (ICH, 2011) with the strategy for food and feed developed by the European Food Safety Authority (EFSA) (EFSA, 2011). For references of the assays: OECD TG 471 (OECD, 2020d), OECD TG 473 (OECD, 2016a), OECD TG 487 (OECD, 2016c), OECD TG 490 (OECD, 2016d), OECD TG 474 (OECD, 2016e), OECD TG 475 (OECD, 2016f), OECD TG 489 (OECD, 2016g) and OECD TG 488 (OECD, 2013).

An exceptional situation is the one regarding nanomaterials (NMs), including nanoparticles (NPs), as its reduced size confers them special physicochemical properties compared to larger size substances. Indeed, it has been demonstrated that the bacterial reverse mutation assay is not suitable for NPs, as bacteria cannot internalize them (Doak *et al*., 2012), and the OECD does not recommend the use of the assay for investigating the genotoxicity of nanomaterials (OECD, 2014). For this reason, EFSA published in 2018 a guidance for the risk assessment of nanomaterials, including a strategy for genotoxicity testing (EFSA, 2018).

2.3. Adverse Outcome Pathways

The Adverse Outcome Pathway (AOP) concept was initially introduced in 2010 as a tool to support risk assessment in ecotoxicology by understanding the mechanisms linking initial interaction(s) of a stressor with adverse effects (Ankley *et al*., 2010). Currently, this new pragmatic tool is in the origin of a paradigm shift in the toxicological evaluation of all kind of chemicals: moving from classical toxicology (focused on apical endpoints) to a more human-relevant mechanistic toxicology. Although more developed in the pharmaceutical and cosmetic sector, its application in food safety is also starting to evolve.

An AOP is a conceptual construct compiling and managing existing knowledge concerning the linkage between a molecular initiating event (MIE; *e.g.,* DNA alkylation) and an adverse outcome (AO; *e.g.,* heritable mutations or cancer). Generally, AOPs are a sequence of events

encompassing multiple levels of biological organization, from molecular over sub-cellular, cellular to organ and organism or, in ecotoxicology, even population level. Thus, after a MIE, there is a progression through several measurable biological changes, known as key events (KE; *e.g.,* alterations of metabolic pathways, signaling events or modifications of cell functions), ultimately leading to the AO (*Figure 3*) (Ankley *et al*., 2010; Leist *et al*., 2017). The OECD published a guideline for developing and assessing AOPs (OECD, 2018), in which it was stablished that each AOP should have one MIE and one AO, but with no limitation in the number of KE. Indeed, several KE can be related in different ways: unidirectional, non-unidirectional, lineal, ramified and KE can even modulate other KE. Causal and predictive connections between KE are known as key event relationships (KER) (*Figure 3*). Moreover, AOPs can share one or more KEs, thereby assembling an AOP network (OECD, 2018).

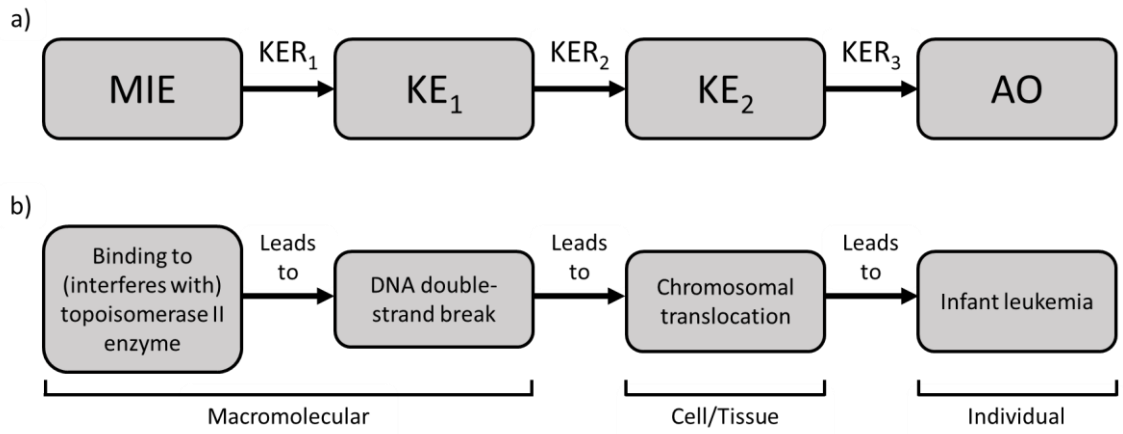

Figure 3. a) Schematic diagram of an AOP, although only 2 KE are represented there is no limitation on its number (adapted from OECD, 2018); b) Example of an AOP describing critical steps at different biological organization levels, from the binding of a toxicant to topoisomerase II to an infant leukemia (AOP no. 202, adapted from AOPWiki, 2019). MIE: molecular initiating event; KE: key event; AO: adverse outcome; KER: key event relationship.

In general, the AO considered in AOPs are the classical endpoints used for toxicity assessment. However, the overall aim of AOPs is also to support risk assessment by describing and linking existing knowledge on mechanisms of toxicity (KE) to an AO. Thus, AOPs pose KE as relevant factors and potential endpoints in decision-making processes for hazard identification (Leist *et al*., 2017). At this point, it is important to clarify that the AOP concept was also developed in response to uncertainties in the use of the term *mechanism of action* and *mode of action*. The first is a detailed description of some of the molecular events taking part between an initiating event and an adverse outcome, whereas the second is reduced to a general description of the outcome and/or initiating event. The AOP concept requires an anchor to both, a molecular initiating event and an adverse outcome, developing the mechanisms underlying the process, being more alike to the mechanism of action concept (Ankley et al., 2010; Vinken, 2013).

The use of AOPs as a framework of knowledge based on mechanistic reasoning to support chemical risk assessment has gained popularity during last years. It is considered a pragmatic new tool that provides numerous opportunities (reviewed in Leist et al., 2017). Indeed, its systematic, structured, dynamic, and quality-controlled collection of weight of evidences simplifies its application. Furthermore, as mentioned before, the AOP concept is supported by the OECD, thereby facilitating its adoption and implementation by regulatory authorities.

In June 2020, the AOP knowledge base maintained by the OECD lists 284 AOPs (with 2087 KE) (OECD, 2020e). Some of these AOPs involve genotoxic mechanisms, such as the AOP 15 "Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations" (AOPWiki, 2020a) or AOP 293 "Increased DNA damage leading to increased risk of breast cancer" (AOPWiki, 2020b). Additionally, several KE related to effects in DNA have been included, such as KE 97 "DNA alkylation", KE 1596 "Oxidation of DNA" , KE 1461 "DNA double-strand break" (shown in *figure 3*) and KE 1636 "Chromosomal aberrations" (AOPWiki, 2020c).

3. The comet assay

3.1. History and principle of the method

The origins of the comet assay can be found back in the 1970s. Particularly in 1976, when Cook and collaborators developed a method to characterize nuclear structure (Cook *et al*., 1976). The basis of this approach was the lysis of cells with nonionic detergent and a high-molarity sodium chloride solution to remove membranes, cytoplasmic components and to disrupt DNA structure (*i.e.,* disrupt the nucleosomes and solubilizing almost all histones). The treatment only leaved nuclear bodies consisting of the supercoiled DNA, arranged as a series of loops being each of which a structural unit, attached at intervals to a matrix composed of RNA and proteins. This structure was called nucleoid. Cook and colleagues observed that the negative supercoiling of DNA in the nucleoid survived as long as DNA was intact. When the supercoiling in a loop was interrupted by a SSB, the loops expanded forming a "halo" of unwind DNA.

A few years later, the comet assay (though not referred to by that term then), or single cell gel electrophoresis, was conceived by Östling and Johanson in 1984 as a technique to measure DNA SBs at the level of individual cells (Östling and Johanson, 1984). For this purpose, and based on the nucleoid theory, they lysed agarose-embedded cells and then applied an electrophoretic field to the nucleoids in a solution at pH 9.5. Thus, they described the migration of DNA towards the anode as a result of the relaxation of the supercoiled loops when cells were γ-irradiated. However, this protocol was not widely adopted.

In 1988, the assay was modified by increasing the alkalinity of the electrophoresis solution (pH>13) by Singh and colleagues (1988). Although during the following years there have been significant modifications, the protocol of that "new" alkaline version is the most widely used nowadays, being since then referred to as comet assay. Indeed, the technique was called comet assay because the outcome of damaged DNA looks like the cosmic bodies (*Figure 4*). The comet assay under alkaline conditions, in addition to SSBs and DSBs, is able to detect alkali-labile sites (ALS), such as AP-sites or baseless sugars.

The protocol of the assay is relatively simple. In brief, cells are embedded in agarose on a microscope slide (or on a plastic film) and lysed with a solution containing a detergent and high concentration of salt (*i.e.,* Triton X-100 and 2.5 M NaCl respectively) at pH 10 (Azqueta and Collins, 2011 and 2013). During lysis, all cell membranes, soluble cytoplasmic and nuclear components (including histones) are removed, leaving the nucleoids. Then, supercoiled DNA in the nucleoids is subjected to alkaline unwinding. During this process, the transformation of ALS into SBs occurs. Then, an electrophoretic field is applied thereby attracting DNA towards the anode. Thus, if DNA integrity is disrupted by an SB, relaxed DNA around this point will migrate further towards the positive pole, creating what is commonly known as the comet tail (*Figure 4*). Therefore, the amount of DNA in the comet tail is proportional to the amount of DNA breaks, providing a measure of DNA damage. For the quantification of the DNA damage on each cell of DNA SBs on each cell, nucleoids are stained with a fluorochrome and observed under a fluorescence microscope. Usually, the DNA migration of 100 comets per condition is analyzed. This can be performed either visually or, more commonly, using image analysis software (semiautomated or automated).

There are different parameters to describe the DNA migration in comets, such as percentage of DNA in tail (*i.e.,* the relative intensity of tail fluorescence) or tail moment, among others; being percentage of tail DNA the most widely used (Møller *et al*., 2014). Additionally, several researchers also express DNA damage in terms of DNA breaks frequency as breaks per cell or breaks per 10^6 base pairs. However, for this purpose it is necessary to perform a calibration curve using cells exposed to ionizing radiation, so an extrapolation of the percentage of DNA in tail can be done (1 Gy of X- or ɣ-irradiation introduces 0.31 breaks per 10^9 Da of DNA) (Ahnström and Erixon, 1981; Collins *et al*., 2008).

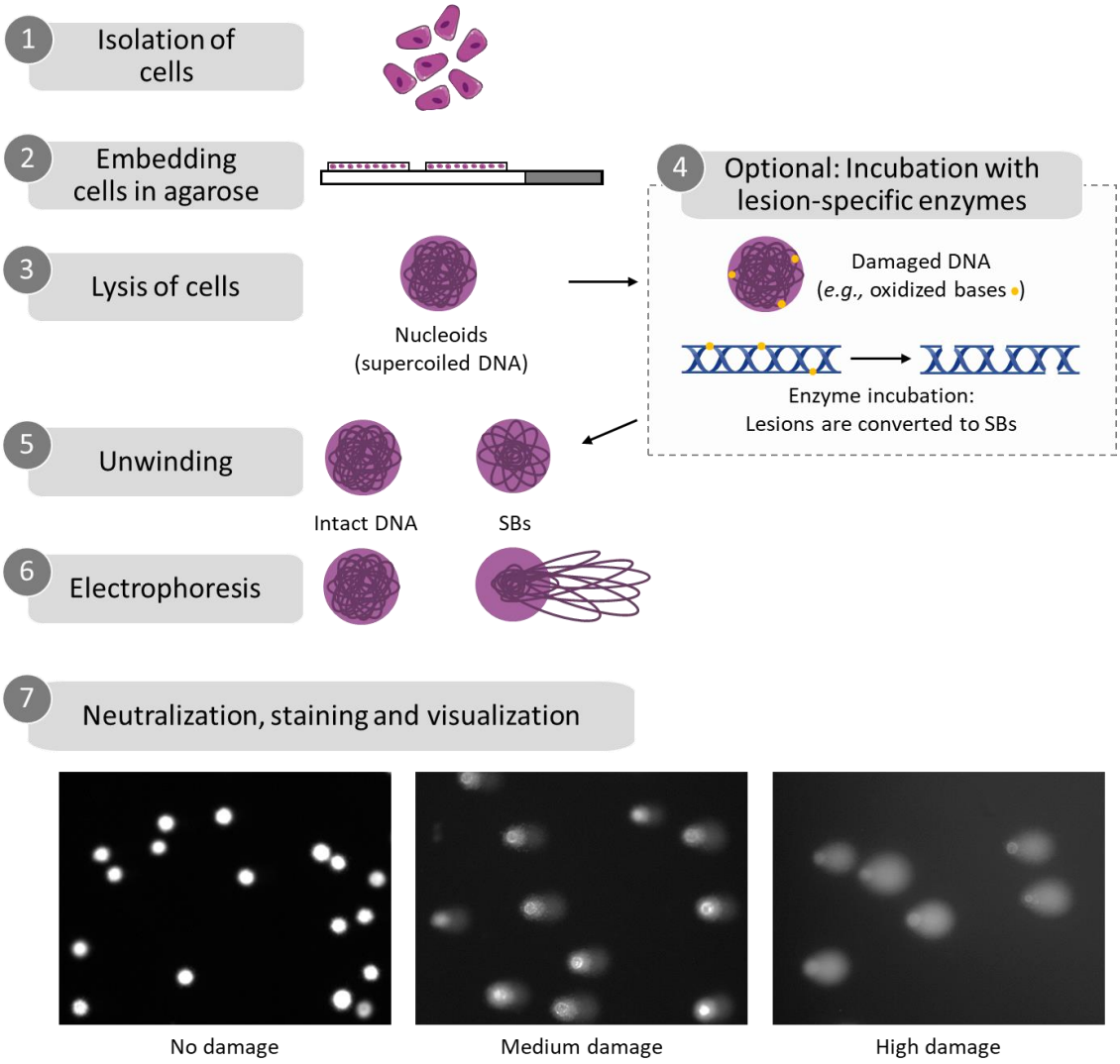

Figure 4. Scheme illustrating the protocol for the standard comet assay and its modified version for the detection of altered bases by inclusion of a specific nuclease digestion step (*i.e.,* enzyme-modified comet assay) following lysis. This modification using enzymes is covered in the section 3.3 of this thesis. The figure was elaborated based on Azqueta and Collins (2011 and 2013). The comet images shows TK-6 cells treated with two concentrations of hydrogen peroxide to induce different levels of DNA damage.

3.2. Advantages and disadvantages

Nowadays, the use of the comet assay for detecting DNA damage has been widely spread throughout the scientific community. Indeed, although its most common application is *in vitro* and *in vivo* genotoxicity testing (to screen drugs, cosmetics, chemicals, etc.), there are many scientific fields in which the comet assay is used. For instance, the assay is widely used in human biomonitoring (including the study of occupational/environmental exposure to hazardous agents, nutritional studies and to assess background levels of damage), ecological monitoring and also in mechanistic toxicology to understand the mechanisms of DNA damage and repair.

The increasing popularity of the comet assay in different research areas can be explained by several reasons. First, it is worth mentioning that in addition to its relatively simple protocol and lack of complex processing, the assay is not very time-consuming (if scoring of the samples is not taken into account), it is an economical assay in terms of materials and reasonably easy to incorporate in almost every laboratory, as the equipment needed to perform the assay can be commonly found in any research laboratory (an electrophoresis tank, a power supply and a fluorescence microscope).

Nevertheless, probably, on top of the advantages it is the versatility and flexibility of the assay. Indeed, the comet assay can be potentially applied to any eukaryotic cell type, including non-dividing cells and tissues, from which single cells or nuclear suspensions can be obtained. Additionally, small number of cells is required (Azqueta and Collins, 2013). Besides, as aforementioned, the DNA damage is measured at the level of individual cells, allowing to detect cell populations with different levels of DNA damage. Finally, the comet assay is a sensitive method for measuring DNA damage; it is capable of detecting damage likely to be found in control cells as a normal background, as well as damage that is inflicted experimentally without killing the cells (*i.e.,* subtoxic concentrations). Indeed, its detection level goes from a few hundred to several thousand DNA breaks per cell (Collins *et al*., 2008).

However, it should be pointed out that the assay also presents some limitations, although it is possible to overcome most of them. One of the most well-known limitation of the assay is its variability. Although there are different levels of variability (*e.g.,* intra-experimental, inter-experimental or inter-laboratory), the variability between laboratories (inter-laboratory) is the most reported one (Forchhammer *et al*., 2010, 2012; Ersson *et al*., 2013; Godschalk *et al*., 2014). A lot of effort has been (and is still being) done to reduce this variation, which in most cases is explained because of differences in the protocol employed on each laboratory. In this regard, the identification of the critical steps of the procedure and the factors influencing the outcome of the assay (Azqueta *et al*., 2011; Ersson and Möller, 2011), the inclusion of reference standards (Møller *et al*., 2020) or the standardization of the assay protocol (OECD, 2016g; Azqueta *et al*., 2019) are different actions carried out to reduce the variability of the comet assay.

Other issue of the comet assay is related with the scoring. The comet assay users know that it is one of the most tedious tasks and, usually, the “bottleneck” of the experiment. In addition, the comet assay has been considered by many as a subjective assay and prone to bias, as comets to be scored and quantified are normally selectively chosen by the user. However, this is easily overcome by scoring coded slides for a blind process. Moreover, in the last years different

automated scoring methods, in which all comets in a sample are evaluated, have been developed (*e.g.*, Pathfinder™ ScreenTox Auto-COMET developed by IMSTAR or Metafer™ by MetaSystems). Despite these resources still need to be improved to increase its accuracy, the throughput and power of these tools turn to be very useful, as they increase the efficiency of the assay considerably. Nevertheless, the high price of these resources significantly reduces its use, at least at research level.

In this line, a practical limitation of the comet assay is its throughput, as the number of samples that can be included in a single experiment is quite low. Indeed, the number of samples is determined by the size of the electrophoresis tank. Particularly, using a standard electrophoresis tank and 2 large gels per slide, only 40 gels can be run at the same time, as 20 slides are the maximum that the electrophoresis tank can hold. However, higher throughput methods have been developed to increase substantially the number of samples that can be handled per experiment. For instance, in 2010 Shaposhnikov and colleagues increased the efficiency of the assay using a medium-throughput format in which 12 mini-gels are set on a microscope slide and can be isolated for individual treatment (Shaposhnikov *et al*., 2010). Other commonly employed technology to increase the throughput of the assay are the plastic Gelbond® films. It was firstly applied in the comet assay by McNamee *et al*. (2000) by including 12 gels per film and 4 films per electrophoresis tank. Then the throughput was extended to 48 and 96 minigels per Gelbond® film, meaning that almost 400 samples could be electrophoresed in a standard tank (Gutzkow *et al*., 2013). Similarly, other methodologies have been also applied, such as 96-well multichambered plates (Stang and Witte, 2009) and even ultrahigh-throughput approaches with agarose-based microfluidic array chips (Li *et al*., 2013); though these are not very used. In 2014, a high-throughput screening platform to perform comet assay, called CometChip was developed (Watson *et al*., 2014). The platform is based on an agarose gel containing microwells, in which single cells are allocated. A total of 300 microwells comprises the bottom of a macrowell (of a 96-well size), which are isolated from each other using a bottomless 96-well plate. Thus, 96 conditions can be readily imaged and analyzed, as cells are patterned creating an array format. Overlapping of comets is avoided and automated scoring systems can easily be applied to scan the sample (Watson *et al*., 2014).

The advantages of running many samples simultaneously are clear, however, the number of samples to be analyzed increase too, leading to the aforementioned limitation of the scoring. Such reasons urge to develop affordable automated scoring systems.

As previously mentioned, the comet assay in its alkaline version, detects SBs and ALS. However, DNA damage tends to appear in different forms, such as oxidized and alkylated bases, adducts and cross-links, among others. These lesions are very relevant in terms of DNA integrity, as many are potentially mutagenic. Fortunately, there are different strategies available in which the comet assay protocol is modified to detect some of these relevant lesions, thereby overcoming its specificity limitation. They are covered in the next section.

3.3. Modifications of the comet assay

The comet assay can be modified to detect not only SBs and ALS, but also other DNA lesions such as oxidized and alkylated DNA bases, cross-links and adducts. There are several modifications that can be applied for detecting these lesions. In this section, two approaches, to detect altered bases (section 3.3.1 Use of enzymes) and other to detect interstrand cross-links (ICL) (section 3.3.2. Modified comet assay detecting cross-links) are described.

Other modification, that will not be covered in this thesis, is the use of DNA repair inhibitors, such as aphidicolin, hydroxyurea and 1-β-D-arabinofuranosylcytosine, that allows the detection of lesions repaired by the nucleotide excision repair pathway (NER), such as bulky adducts, which are not detected with the others modifications (Gedik *et al*., 1992; Martin *et al*., 1999; Speit *et al*., 2004; Güerci *et al*., 2009; Vande Loock *et al*., 2010; Ngo *et al*., 2020).

It is also worth mentioning other modifications and variants of the comet assay which are intended to increase the applicability of the method rather than the range of lesions that can be detected. For instance, the comet assay can be used for measuring DNA repair activity (reviewed in Azqueta *et al*., 2014) and global methylation status (Wasson *et al*., 2006; Wentzel *et al*., 2010; Georgieva *et al*., 2017). Moreover, it is also possible to combine the comet assay with fluorescent *in situ* hybridization (FISH) for studying damage and repair at the level of genes (reviewed in Shaposhnikov *et al*., 2009).

3.3.1. Use of enzymes

Most small base alterations that do not significantly distort the DNA helix structure (*e.g*., DNA oxidations and alkylations, or deaminations like the presence of uracil in DNA) are repaired by the base excision repair pathway (BER) (reviewed in Krokan and Bjoras, 2013). BER pathway is initiated by lesion-specific glycosylases which are responsible of detecting and removing the damaged base leaving an AP-site, which will be subsequently processed to a SB, filled, and finally ligated. In the context of the comet assay, nucleoids obtained after lysing the cells can be digested with some of these lesion-specific glycosylases thereby creating AP-sites (or directly a

break if the enzyme presents AP-lyase activity), which can be detected following the rest of the comet assay protocol (*Figure. 4*). Several lesion-specific enzymes from the DNA repair machinery of bacteria and humans have been used in combination with the comet assay allowing the detection of different oxidized and alkylated bases as well as cyclobutane pyrimidine dimers, mis-incorporated uracil and AP-sites. A full review of all the enzymes that have been combined with the comet assay is included in Chapter 3.

3.3.2. Modified comet assay for detecting cross-links

The comet assay has been modified for the detection of cross-links by measuring the reduction of DNA tails. Indeed, two approaches for cross-links detection were described two decades ago (Pfuhler and Wolf, 1996; Tice *et al*., 1997) as it was shown that cross-links inhibit DNA migration during electrophoresis in the comet assay (*i.e.,* having the opposite effect of SBs) (Olive *et al*., 1992). One of them consists in the detection of cross-links by increasing the duration of the electrophoresis to such an extent that even DNA of non-treated cells migrates considerably (Tice *et al.*, 1997). Thus, DNA containing cross-links will migrate less compared to DNA of control cells. However, the use of this approach has not been extended.

Alternatively, control and treated cells can be exposed to a second genotoxic agent, either chemical (*e.g.,* hydrogen peroxide) or physical (*e.g.,* ionizing radiation), for inducing a known number of DNA breaks (or a known amount of DNA damage in terms of % tail intensity). Therefore, the reduction of normal migration of DNA after the treatment with the DNA breaking agent, when comparing cells treated with the potential cross-linking agent with control cells, is determined (Olive *et al*., 1992; Tice *et al*., 2000). This modification has been the most used.

Cross-links can involve either DNA molecules (intra- or inter-strand cross-links) or a DNA molecule and a protein. The modified version of the comet assay has been applied to detect all of these cross-links (Pfuhler and Wolf, 1996; Merk and Speit, 1998). However, most recent publications are focused on the detection of inter-strand cross-links (Spanswick *et al*., 2010; Wu and Jones, 2012; Swift *et al*., 2020).

This modification of the comet assay has been also applied to evaluate not only the presence but also the repair of DNA-DNA cross-links (Blasiak *et al*., 2000; Jost *et al*., 2015; Swift *et al*., 2020). For instance, it has been applied in human lymphocytes for measuring DNA damage and repair after exposure to anticancer platinum drugs (Błasiak *et al*., 2000). Likewise, Jost and collaborators evaluated the induction and repair of ICL after sulfur mustard treatment (Jost *et al*., 2015).

However, the use of this modification have been significantly less extended compared to the use of enzymes combined with the comet assay.

3.4. Role in regulatory toxicology

3.4.1. The *in vivo* comet assay

The *in vivo* comet assay was included as a complementary assay following positive *in vitro* results, to evaluate target organ-specific genotoxicity, or as a second *in vivo* test within different test batteries for risk assessment of different regulatory agencies in the early 2000s (reviewed in Brendler-Schwaab *et al*., 2005). The great value in this context is that the comet assay can be applied to any tissue (Azqueta and Collins, 2013).

After the development of internationally agreed protocols, a formal validation of the *in vivo* comet assay was performed in 2006-2012. The trial was coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with EURL-ECVAM, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (Uno *et al*., 2015a, 2015b). The validation of the assay promoted the publication of the OECD testing guideline 489 "*In vivo* Mammalian Alkaline Comet Assay" in 2014, and its last version was adopted in 2016 (OECD, 2016g), although it should be noted that the OECD only validated the standard comet assay with no modifications to its protocol.

As aforementioned, nowadays the *in vivo* comet assay is part of several strategies for genotoxicity testing suggested by different regulatory agencies, such as ICH and EFSA (*Figure 2*). While the ICH include the only the comet assay on its standard version and on liver, the EFSA considered it as a follow up test that can be carried out in any tissue and mentions the use of enzymes for the detection of oxidized bases (ICH, 2011; EFSA, 2011).

Furthermore, some testing strategies have promoted the integration of genotoxicity tests. This is the case of the micronucleus and the comet assays; the complementary use of different target organs and genetic endpoints, in addition to similar experimental requirements while reducing the number of testing animals, strongly support the combination of these two assays (Speit *et al*., 2015). Moreover, it has also been proposed to integrate the comet assay, within other toxicological assays (*e.g.,* a 28-day tolerance study or repeated-dose toxicity study), such as the one proposed by the European Chemical Agency (ECHA) (ECHA, 2017). Indeed, as an attempt to improve the genotoxicity assessment while implementing three Rs principle, it was proposed

the combination and integration of the *in vivo* micronucleus test and the *in vivo* liver comet assay within acute and repeated dose studies (Rothfuss *et al*., 2011; Corcuera *et al*., 2015; Frötschl, 2015).

3.4.2. The *in vitro* comet assay

The *in vitro* version of the alkaline comet assay has not an OECD testing guideline, although an international validation study was proposed in the Fourth International Workgroup on Genotoxicity testing (IWGT) (Burlinson *et al*., 2007). However, the validation study leaded by JaCVAM is currently stopped (EURL-ECVAM, 2019) and the *in vitro* comet assay is not considered within the standard genotoxicity battery tests. Indeed *in vitro* tests employed to detect classical regulatory endpoints (*e.g.,* gene mutation or chromosomal mutations) have been long established and implemented in regulatory guidelines (Frötschl, 2015). In this regard, the standard comet assay might not give value in risk assessment compared to other *in vitro* genotoxicity assays nor to the *in vivo* version.

However, the use of the *in vitro* comet assay is widely extended in research for testing novel substances such as NMs, drugs, and cosmetics. Indeed, EFSA recommends the use of the *in vitro* comet assay to provide complementary information of the genotoxic mechanisms of action of nanomaterials, especially the enzyme-modified version for the detection of oxidized DNA, as many NMs have been shown to induce oxidative stress (EFSA, 2018). Furthermore, since the European regulation ("Regulation (EC) No 1223/2009 Of the European Parliament and of the Council of 30 November 2009 on cosmetic products") (EC, 2009) prohibited the use of animal testing for cosmetic ingredients, the *in vitro* comet assay applied to *in vitro* models of human skin arise as a good alternative. Indeed, the combination of the *in vitro* comet assay and a 3D skin model was validated in a study lead by Cosmetics Europe with the support of EURL-ECVAM and is currently in the OECD Test Guideline Programme (OECD TGP) work plan (EURL-ECVAM, 2019).

3.5. Future perspectives: *In vitro* comet assay in mechanistic toxicology

As seen in the section 2.3, one of the current trends in toxicology is to consider mechanistic toxicological data as relevant factors and potential endpoints in risk assessment, as mechanistic endpoints might be linked to classical and apical endpoints (*e.g.*, gene mutation to carcinogenesis). Thus, the traditional concept of mechanistic information as a supplement to explain and rationalize apical endpoints of toxicity is completely changing. Furthermore, the development of mechanistic-based tools, such as AOPs, have been of value in driving the

development and application of non-animal approaches to hazard and risk assessment (Burden *et al*., 2015; Sewell *et al*., 2018). Therefore, *in vitro* and *in silico* test systems are about to reach a new level of relevance in risk assessment (Leist *et al*., 2017). In this new context, the potential of the *in vitro* comet assay lies in the modifications of the protocol to detect other lesions than SBs and ALS (*e.g.,* oxidized bases or cross-links).

Concerning AOPs, *in vitro* and/or *in silico* methods integrated within a test battery, based on or linked to KE in relevant AOPs, will be more likely to gain regulatory acceptance than tests not linked or supported by an AOP (Leist *et al*., 2017). In this context, the comet assay arise as a good candidate, as its versatility and the possibilities for its modification (*e.g.,* combination with DNA repair enzymes) allow the screening of different mechanisms of action in the field of genotoxicity (*e.g.,* SBs, oxidized or alkylated bases). Indeed, some of these mechanisms of action detected by the comet assay are already registered as KE in the AOPWiki (*Table* 2).

Table 2. KE that can be measured using the comet assay and the AOPs in which they are included. Information was obtained by full-text searching "comet assay" in the KE section of the AOPWiki (AOPWiki, 2020c).

KE ID	KE Title	AOP(s) including the KE (short name)	AOP ID
97	Alkylation, DNA	Alkylation of DNA leading to heritable mutations	15
		DNA alkylation -> cancer 1	139*
		DNA alkylation -> cancer 2	141
		Alkylation of DNA leading to reduced sperm count	322**
155	Inadequate DNA repair	Alkylation of DNA leading to heritable mutations	15
		DNA alkylation -> cancer 1	139*
		DNA alkylation -> cancer 2	141
		Oxidative DNA damage, chromosomal aberrations and mutations	296**
		Ionizing energy leading to lung cancer	272**
		Alkylation of DNA leading to reduced sperm count	322**
1194	Increase, DNA damage	ER activation to breast cancer	200*
		ROS production leading to reproduction decline	216*
		Increased DNA damage leading to breast cancer	293**
		RONS leading to breast cancer	294**
1252	Binding to (interferes with) topoisomerase II enzyme	Topoisomerase II binding, infant leukaemia	202**
1253	MLL chromosomal translocation	Topoisomerase II binding, infant leukaemia	202**
1634	Increase, Oxidative damage to DNA	Oxidative DNA damage, chromosomal aberrations and mutations	296**
1635	Increase, DNA strand breaks	Oxidative DNA damage, chromosomal aberrations and mutations	296**
		Ionizing energy leading to lung cancer	272**
		Alkylation of DNA leading to reduced sperm count	322**
1669	Increased, DNA damage and mutation	Frustrated phagocytosis-induced lung cancer	303*

*Under development **Draft under review. Abbreviations: ER, estrogen receptor; ROS, reactive oxygen species; RONS, reactive oxygen and nitrogen species; MLL, mixed-lineage leukaemia.

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Chapter 2

Aim and objectives

1. Aim

The main aim of this thesis was to develop and validate a new tool for *in vitro* genotoxicity testing, based on the comet assay, that can be used for the elucidation of different genotoxic mechanisms of action and may represent a good candidate for complementing current *in vitro* genotoxicity testing batteries.

2. General considerations

Many studies have been carried out to evaluate the effect of different comet assay conditions, such as agarose concentrations, lysis duration or the duration of electrophoresis and the voltage gradient applied. In the case of the enzyme-modified comet assay, the effects of the enzyme concentration and incubation time have also been reported. Nevertheless, the enzyme incubation conditions (*i.e.*, enzyme concentration and incubation time) are copied from one laboratory to another rather than adapted, thereby ignoring the differences in other assay conditions or even the comet assay format used (*e.g.,* 2 gels/slide, 12 minigels/slide or 96 minigels/Gelbond® film), which affect the way the incubation is performed. This may increase the inter-laboratory variation of the enzyme-sensitive sites detected.

On the other hand, the *in vitro* comet assay has been widely used in combination with different enzymes, mainly for the detection of oxidized DNA bases, being formamidopyrimidine DNA glycosylase (Fpg) the most used one. Moreover, it is also known that Fpg detects ring-opened purines (*i.e.,* formamidopyrimidines, commonly known as Fapy). For this reason, this enzyme can also detect some alkylated bases when combined with the comet assay as some of these lesions are transformed into ring-opened purines during the lysis step of the assay due to the alkaline pH. The assay has also been combined with 3-methyladenine DNA glycosylase II (AlkA) and 3-methyladenine DNA glycosylase (AlkD) for the detection of alkylated bases. However, the use of these enzymes in combination with the comet assay has been used rarely as the enzymes are not commercially available.

Finally, there is only an OECD guideline for the *in vivo* comet assay on its standard version. Regarding the *in vitro* comet assay, its main potential lies on the use of modifications to detect different DNA lesions, thereby adding great value to the study of mechanisms of action. A combination of the comet assay with different enzymes, as well as its modification for cross-links detection may have a crucial role in regulatory context.

3. Specific objectives

The specific objectives of this thesis were:

1. To prepare a review of all the enzymes that have been used in combination with the comet assay (**Chapter 3**).
2. To study the effect of the enzyme incubation conditions in the outcome of the Fpg-modified comet assay when using different throughput formats (**Chapter 4**).
3. To set up the use of the commercially available enzyme human alkyladenine DNA glycosylase (hAAG) in the comet assay, for the detection of alkylated DNA bases and compare its performance with the enzyme Fpg (**Chapter 5**).
4. To perform an internal validation of the enzyme-modified comet assay, using different enzymes to detect oxidized and alkylated bases, and the comet assay modified for the detection of cross-links (**Chapter 6**).

Chapter 3

The enzyme-modified comet assay: Past, present and future

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Review

The enzyme-modified comet assay: Past, present and future

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The enzyme-modified comet assay was developed in order to detect DNA lesions other than those detected by the standard version (single and double strand breaks and alkali-labile sites). Various lesion-specific enzymes, from the DNA repair machinery of bacteria and humans, have been combined with the comet assay, allowing detection of different oxidized and alkylated bases as well as cyclobutane pyrimidine dimers, mis-incorporated uracil and apurinic/apyrimidinic sites. The enzyme-modified comet assay has been applied in different fields - human biomonitoring, environmental toxicology, and genotoxicity testing (both in *vitro* and in *vivo*) - as well as in basic research. Up to now, twelve enzymes have been employed; here we describe the enzymes and give examples of studies in which they have been applied. The bacterial formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (EndoIII) have been extensively used while others have been used only rarely. Adding further enzymes to the comet assay toolbox could potentially increase the variety of DNA lesions that can be detected. The enzyme-modified comet assay can play a crucial role in the elucidation of the mechanism of action of both direct and indirect genotoxins, thus increasing the value of the assay in the regulatory context.

1. The enzyme-modified comet assay

The alkaline comet assay (single cell gel electrophoresis) is a widely used method for measuring DNA damage at the level of individual cells – whether endogenous damage, or damage induced by exogenous agents, such as chemicals, radiation and nanomaterials (NMs) (Azqueta and Collins 2013; Møller et al., 2015; Neri et al., 2015). The assay is applicable to any eukaryotic cell type, and also to disaggregated tissues from which single cells or nuclear suspensions can be obtained. The comet assay can be applied in different areas such as human and environmental biomonitoring, *in vitro* and *in vivo* genotoxicity testing of chemicals and NMs, and in ecotoxicity studies, including applications to plants (Brendler-Schwaab et al., 2005; Witte et al., 2007; Azqueta and Dusinska, 2015; Møller et al., 2015; Santos et al., 2015; OECD, 2016; Gajski et al., 2019a; 2019b; Azqueta et al., 2020).

As first devised by Östling and Johansson (1984), the method involved lysis of agarose-embedded cells with detergent and high NaCl, at near-neutral pH, to remove cell membranes and soluble components, and also histones, leaving DNA attached to the nuclear matrix as nucleoids. If cells were γ-irradiated to introduce breaks in the phosphodiester backbone, on electrophoresis and staining with acridine orange, a 'tail' of DNA was seen extending from each nucleoid core towards the anode, the intensity of the tail fluorescence increasing with radiation dose. This was explained by the authors as due to the relaxation of supercoiled lengths of DNA, making them free to migrate; the more strand breaks (SBs) there were, the more DNA appeared in the tail (Östling and Johanson, 1984). Note that both single- and double-strand breaks (SSBs and DSBs, respectively) will release supercoiling, and so, unlike other methods, the assay does not depend on alkaline unwinding to make SSBs visible. The alkaline version of the assay was developed a few years later (Singh et al., 1988), and this is the method most often used at present. Because of the higher pH, in addition to frank SBs, alkali-labile sites (ALS) (e.g. apurinic/apyrimidinic [AP-] sites or base-less sugars) are also detected. Comets are observed by fluorescence microscopy and scored visually or (usually) using image analysis software.

Many DNA-damaging agents do not directly induce SBs but cause other lesions, such as oxidized or alkylated bases, bulky adducts, and intra- and inter-strand cross-links, which tend to have more serious consequences for the cell or organism than readily reparable single SBs, but are not detected by the standard comet assay. To overcome this restriction, the comet assay has been modified using different approaches. For instance, to measure inter-strand cross-links, advantage is taken of the fact that cross-links block the migration of DNA that has

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been broken by ionising radiation, so the less intense the comet tails, the more cross-linking is present - an inverse comet assay, so to speak (Olive et al., 1992; Danson et al., 2007). The DNA synthesis inhibitors aphidicolin, hydroxyurea and 1-β-D-arabinofuranosyl cytosine, or different combinations, have been applied (though not widely) for the detection of bulky adducts or UV-induced lesions; these compounds inhibit the DNA synthesis step of nucleotide excision repair (NER) inducing the accumulation of breaks (Miller et al., 1996; Ngo et al., 2020).

This review deals with a modification that has been particularly popular and productive – the use of lesion-specific endonucleases to detect different DNA lesions. The enzyme is applied after lysis, directly to the nucleoids embedded in the agarose. During an incubation period, the enzyme induces extra breaks (or AP-sites), and these are measured by continuing with the standard assay protocol. As a control, nucleoids are incubated with the enzyme buffer but without enzyme; subtracting the score obtained in this sample from that with the enzyme gives the frequency of "net enzyme-sensitive sites". The first such modification (Collins et al., 1993) was the use of the bacterial repair enzyme endonuclease III (EndoIII), which makes breaks at sites of oxidized pyrimidines, and it was very soon applied to human biomonitoring in the area of nutritional intervention trials (Duthie et al., 1996).

Here we provide an overview of the enzymes that have been used in combination with the comet assay to measure different DNA lesions: endonuclease III (EndoIII), formamidopyrimidine DNA glycosylase (Fpg), uvrABC, T4 endonuclease V (T4endoV), uracil DNA glycosylase (Udg), exonuclease III (ExoIII), 3-methyladenine DNA glycosylase II (AlkA), 8-oxoguanine DNA-glycosylase (hOGG1), endonuclease IV (EndoIV), endonuclease III-like protein 1 (NTH1), 3-methyladenine DNA glycosylase (AlkD) and alkyladenine DNA glycosylase (hAAG).

Certain enzymes have been widely used, and others more rarely. In some cases, the name of the enzyme includes the enzyme's substrate, but this can be misleading. A good example is Fpg, which was found to act at oxidized purines and not just on the formamido-products (ring-opened purines) (see section 4 below). The broad specificity of Fpg (detecting also ring-opened purines resulting from the breakdown of alkylated bases) can be seen as an advantage if the aim is to detect as many lesions as possible, but care needs to be taken in interpreting results. Several enzymes have the ability to make breaks at alkali-labile sites, which, while not their main substrate, are an intermediate in the DNA damage removal. When describing enzymes, we have included their main substrates. This is not a review of all the studies in which the enzyme-modified (or enzyme-linked) comet assay has been employed, but is rather a catalogue of the different enzymes used, with illustrations of the different areas of application. In addition, we will discuss potential future developments and applications of this approach.

2. Enzymes employed with the comet assay

To illustrate the use of the different enzymes, we reviewed papers retrieved from PubMed with the following search strategy: ((comet assay) OR (single cell gel electrophoresis)) AND ((*name of the enzyme*) OR (*abbreviation of the enzyme*)). The names used are the ones described in the previous paragraph, with several versions of the abbreviations (e.g., EndoIII, Endo III and Nth for endonuclease III). The title and abstract of all papers retrieved were checked to ensure that the papers were actually using the comet assay with the corresponding enzymes. The full paper was checked if the information included in the title and abstract were not enough to know if the authors used the comet assay combined with the corresponding enzyme or to determine its correct classification. Thus, the inclusion criterion was papers in which the comet assay was performed in combination with the corresponding enzyme or with other enzyme(s), while the following were excluded: papers in which the enzymes were mentioned but not used in combination with the comet assay, review papers, protocols, and papers in which the enzymes (i.e., Fpg and T4endoV) were used as a control for the comet-based *in vitro* DNA repair assay. A few key papers have been included manually since, surprisingly, they were not retrieved by the electronic search. This is the case of one of the first papers in which Fpg was used with the comet assay, in which the combination with uvrABC was also described, or the first paper in which the comet assay was combined with T4endoV, among others. Similarly, in the case of UDG, we have included several papers, the majority from the research group that set up the assay with this enzyme. Thus, the total number of papers we analysed (before the aforementioned exclusion) was: 268 for EndoIII, 577 for Fpg, 1 for uvrABC, 23 for T4EndoV, 25 for Udg, 7 for ExoIII, 25 for AlkA, 256 for hOGG1, 2 for EndoIV, 2 for NTH1 and 1 for hAAG. Papers were classified in different categories depending on the application of the enzyme-modified comet assay: human biomonitoring studies, including clinical studies; *in vitro* and *in vivo* genotoxicity studies, including DNA protection studies; and ecological studies, including *in vitro* and *in vivo* studies. One paper might contain different types of studies (*in vitro* and *in vivo* studies, for example), in which case it would be classified as both.

The list of enzymes was constructed by the authors through their experience in the development, standardization and use of the comet assay and by performing the aforementioned search. Moreover, a general search was also performed in order to retrieve other potential enzymes in use.

Table 1 shows the total number of papers including the use of each enzyme with the comet assay, and the number of papers per application. The table also shows the first work describing such use, the DNA lesions detected by that enzyme, and the commercial availability according to our knowledge. Enzymes are listed in order of their first use in the comet assay.

*Neutral comet assay. Abbreviations: EndoIII – Endonuclease III -, Fpg – Formamidopyrimidine DNA glycosylase -, T4EndoV – T4 endonuclease V -, Udg – Uracil DNA glycosylase -, ExoIII – Exonuclease III -, AlkA – 3-methyladenine DNA glycosylase II -, hOGG1 – Human 8-oxoguanine DNA-glycosylase -, EndoIV – Endonuclease IV -, NTH1 – Endonuclease III-like protein 1 -, AlkD – 3-methyladenine DNA glycosylase -, hAAG – Human alkyladenine DNA glycosylase -, Eco – Ecological studies -, Fapy – ring-opened purines -, AP-sites – apurinic/apyrimidinic -, COM – Commercially available (currently) -.

As can be seen, EndoIII was the first enzyme to be used in combination with the comet assay. However, Fpg is by far the most used, followed by EndoIII. Half of the enzymes have been used only rarely, appearing in 4 or fewer papers (i.e., uvrABC, ExoIII, EndoIV, NTH1, AlkD and hAAG - though the last has only recently had its first publication). Only 3 of the enzymes are prepared in research laboratories and not commercially available: uvrABC, AlkA and AlkD.

Although it is not reflected in the table, it is worth mentioning that some of the enzymes are quite commonly used together - such as Fpg with EndoIII (149 publications) or hOGG1 with EndoIII (10 publications).

As can be observed in Table 1, the enzyme-modified comet assay is widely applied in *in vitro* and *in vivo* studies, with the aim to study the genotoxicity of different substances, in the majority of the cases. It is also used in human biomonitoring studies and less frequently in environmental and ecotoxicity studies.

There now follows a brief explanation of the functions of each enzyme and its applications. The selection of the examples, in those cases where many publications are available, was subjective, based on the authors' experience, but included key papers, and recent publications. In all cases, the first paper in which the enzyme was used in combination with the assay is mentioned.

3. Endonuclease III (EndoIII)

The enzyme EndoIII, or Nth, also known as thymine glycol DNA glycosylase, is an *E. coli* enzyme involved in the excision of oxidized pyrimidines from double-stranded DNA through its N-glycosylase activity, generating AP-sites. Then, as the enzyme presents AP-lyase activity, the AP-site is cleaved 3' (β elimination) leaving a 5′ phosphate

Table 1
Enzymes used in combination with the comet assay.

Enzyme	EndoIII	Fpg	uvrABC	T4EndoV	Udg	ExoIII	AlkA	hOGG1	EndoIV	NTH1	AlkD	hAAG
No. Of papers included	231	554	1	20	14	4	19	75	2	2	2	1
No. Of papers/ type of study	Human: 60 *In vitro*: 131 *In vivo*: 31 Eco: 11	Human: 116 *In vitro*: 335 *In vivo*: 67 Eco: 41	Human: 0 *In vitro*: 1 *In vivo*: 0 Eco: 0	Human: 3 *In vitro*: 15 *In vivo*: 0 Eco: 2	Human: 6 *In vitro*: 6 *In vivo*: 2 Eco: 0	Human: 0 *In vitro*: 3 *In vivo*: 0 Eco: 1	Human: 5 *In vitro*: 14 *In vivo*: 0 Eco: 0	Human: 18 *In vitro*: 38 *In vivo*: 14 Eco: 5	Human: 0 *In vitro*: 2 *In vivo*: 0 Eco: 0	Human: 2 *In vitro*: 0 *In vivo*: 0 Eco: 0	Human: 0 *In vitro*: 2 *In vivo*: 0 Eco: 0	Human: 0 *In vitro*: 1 *In vivo*: 0 Eco: 0
First paper	Collins et al. (1993)	Evans et al. (1995)	Dusinská and Collins (1996)	Collins et al. (1997)	Duthie and McMillan (1997)	Angelis et al. (1999)	Collins et al. (2001)	Smith et al. (2006)	Holt and Georgakilas (2007)*	Morawiec et al. (2008)	Hasplová et al. (2012)	Muruzabal et al. (2020)
DNA lesions detected	Oxidized pyrimidines	Oxidized purines (specially 8-oxoguanines) and Fapy that results from the breakdown of oxidized and alkylated purines	Cyclobutane pyrimidine dimers and bulky and DNA-distorting adducts	Cyclobutane pyrimidine dimers	Uracil residue and some deamination products	AP-sites	Alkylated bases and hypoxanthine	Oxidized purines (specially 8-oxoguanines) and some Fapy	AP-sites	Oxidized pyrimidines	Alkylated bases	Alkylated bases, ethenoadenines and hypoxanthine
COM	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes

and a 3′-phospho-α, β-unsaturated aldehyde (Doetsch and Cunningham, 1990; David and Williams, 1998). EndoIII is able to recognize a wide range of oxidized pyrimidines, including thymine glycol, 5-hydroxycytosine, uracil glycol, cytosine glycol, and 5-hydroxyuracil (Doetsch and Cunningham, 1990; Boiteux, 1993; David and Williams, 1998). Additional EndoIII substrates (including the purine-derived substrate FapyAde) were identified when specificity studies were performed using GC/MS and DNA with multiple lesions (Dizdaroglu, 2005).

The initial comet assay trial with EndoIII made use of HeLa cells treated with different doses of H_2O_2 and subsequently incubated for a short time to allow the rejoining of SBs, but leaving the more slowly repaired oxidized bases as substrate for the enzyme (Collins et al., 1993). EndoIII was expected to convert oxidized bases into SBs, which could be detected with the comet assay. Results showed an H_2O_2-dependent increase in DNA breaks with the enzyme compared with enzyme buffer alone, while non-treated cells presented no significant increase in DNA breakage after incubation with EndoIII. In the same work, the EndoIII-modified assay was applied to human lymphocytes from healthy individuals to detect the presence of oxidized DNA bases originating *in vivo*. Unlike untreated HeLa cells, lymphocytes from healthy individuals contained numerous sites sensitive to EndoIII. In both HeLa cells and human lymphocytes, increasing enzyme concentration had no effect on comet appearance.

3.1. Human biomonitoring

The authors of the 1993 paper mentioned that EndoIII was already in use in an antioxidant supplementation trial. This nutritional intervention trial – the first to combine an enzyme with the comet assay - demonstrated the effect of antioxidant supplementation on endogenous DNA base oxidation in lymphocytes as well as an enhancement of resistance to exogenous oxidation (Duthie et al., 1996). These results demonstrated the value and convenience of the comet assay for screening populations for DNA damage effects.

Since then, the comet assay in combination with EndoIII has been extensively used in human biomonitoring to evaluate oxidative stress, usually in peripheral blood mononuclear cells (PBMCs, commonly referred to as lymphocytes), and in nutritional intervention trials (Collins, 2017). For example, Moser et al. (2011) evaluated the protective effect of spinach consumption on DNA stability of lymphocytes in a nutritional intervention trial.

In a different context – clinical studies – Devecioglu et al. (2018) evaluated the effect of anastrazole (a third generation aromatase inhibitor, used in the endocrine therapy of breast cancer in postmenopausal women) on oxidation damage in lymphocytes from patients, employing the enzyme-modified comet assay with EndoIII and Fpg. Results showed that anastrozole did not contribute to DNA oxidation damage, as neither EndoIII- nor Fpg-sensitive sites increased in patients using anastrozole compared to a control group (although it should be noted that the study was limited by the small number of samples).

The role of the comet assay in human biomonitoring studies has been recently assessed in a review (Azqueta et al., 2020). The use of EndoIII, Fpg, hOGG1 and Udg in different studies is covered.

3.2. Genotoxicity testing (in vitro and in vivo)

The enzyme has been extensively used for genotoxicity testing using both *in vitro* and *in vivo* experimental systems. For instance, Michalowicz and Majsterek (2010) evaluated for the first time the oxidative modification of DNA purines and pyrimidines in PBMCs by chlorophenols, chlorocatechols and chloroguaicols using Fpg and EndoIII. The oxidation damage induced by low concentrations of chlorinated phenols and chlorocatechols affected mostly pyrimidines, as more EndoIII-sensitive sites were found in comparison with Fpg-sensitive sites.

Similarly, employing the comet assay with repair enzymes including EndoIII, Mokra et al. (2018) showed for the first time that bisphenol A (and other structural analogues) induced DNA oxidation in human PBMCs. More recently, Dalberto et al. (2020) evaluated the genotoxicity of cotinine (the main metabolite of nicotine) and nicotine in a neuronal cell line (SH-SY5Y) using the comet assay in combination with different enzymes (including EndoIII). Results showed a significant increase only in Fpg-sensitive sites in treated cells compared to controls, suggesting that cotinine and nicotine induced oxidized purines rather than oxidized pyrimidines. The *in vitro* comet assay with EndoIII has also been applied to assess the genotoxicity of NMs. For example, Demir et al. (2014) investigated the mechanism of action of two different sizes of zinc oxide nanoparticles (ZnO NPs) in the human TK-6 cell line; they showed that only the effects induced by the larger ZnO NPs (50–80 nm *vs* $\leq$ 35mn) may be attributed to DNA oxidation damage.

In vivo genotoxicity studies have also been performed with the EndoIII-modified comet assay. Ding et al. (2011) evaluated the DNA damage induced in the livers of F344 rats after single oral doses of methyleugenol (MEG). At doses of MEG that produce tumours in rodents, EndoIII-sensitive sites increased significantly after exposure, implying that this might be one of its carcinogenic modes of action. Shukla et al. (2011) positively evaluated the use of the enzyme-modified comet assay for the detection of *in vivo* DNA oxidation damage in *Drosophila melanogaster* treated with well-known oxidizing agents. In a more recent publication, Novotna et al. (2017) assessed the effects of iron oxide and cobalt-zinc-iron NPs, used for labelling and tracking transplanted cells, in rats using the EndoIII- and Fpg-modified comet assay. Their results suggested that implantation of cells labelled with either type of NPs does not induce noticeable oxidative stress in brain tissue of treated rats.

3.3. Environmental biomonitoring and ecotoxicology

The EndoIII-modified comet assay has been applied in other scientific disciplines, such as ecotoxicology. As an example, Iturburu et al. (2018) evaluated the *in vivo* genotoxicity of a neonicotinoid insecticide (imidacloprid) in freshwater fish (*Australoheros facetus*) as a non-target organism. Their results showed oxidation damage in DNA of fishes acutely exposed to environmentally relevant concentrations of the insecticide.

3.4. Other studies

The EndoIII-modified comet assay has also been used to study the DNA protection potential of different compounds. As an example, Kager et al. (2010) evaluated the protective effect of green tea extract consumption against endogenous DNA oxidation damage in healthy rats, showing that high doses of the extracts reduced the basal enzyme-sensitive sites more than 70% in different tissues. Similarly, the chemoprotective capacity of coffee silverskin extract in HepG2 cells was measured with the enzyme-modified comet assay (Iriondo-DeHond et al., 2017); the extract reduced the levels of oxidized purines and pyrimidines (Fpg- and EndoIII-sensitive sites respectively).

4. Formamidopyrimidine DNA glycosylase (Fpg)

Fpg (also known as Mut M) is a DNA N-glycosylase from *E. coli* named after its ability to excises ring-opened purines derived from damaged adenine and guanine (formamidopyrimidines, Fapy), creating AP-sites (Boiteux et al., 1990, 1992). In addition to the base excision function, like other DNA glycosylases, Fpg also possesses an associated AP-lyase activity, which removes the AP-site leaving a 1-base gap. It was later found that probably the main substrates of Fpg *in vivo* are oxidized purines, especially 8-oxoguanine, rather than Fapy residues (Boiteux, 1993).

The enzyme was first used in combination with the comet assay shortly after the introduction of EndoIII by Evans et al. in 1995. They used human promyelocitic cells (HL60), irradiated with white light in the presence of riboflavin. They observed an increase in the tail moment when lysed cells were incubated with Fpg at some of the riboflavin concentrations tested. A year later, Dušinská and Collins (1996), without knowledge of Evans and colleagues' work, published a study aimed at increasing the range and sensitivity of the assay by detecting new DNA lesions using different enzymes: Fpg and uvrABC. They optimized the conditions for the enzyme digestion step in the comet assay. The Fpg-modified comet assay was applied in H_2O_2-treated HeLa cells, just after the treatment and after 2 h of incubation in medium to allow the repair of the DNA SBs, giving a more precise measure of DNA oxidation damage. They also applied the Fpg-modified comet assay in H_2O_2-treated and untreated human lymphocytes from healthy volunteers; they were able to measure the endogenous level of oxidatively damaged DNA. The frequency of Fpg-sensitive sites in human lymphocytes was estimated as 0.25 per 10^9 Da (similar to the frequency of EndoIII-sensitive sites, 0.22) by comparison with X-irradiated cells, for which the break frequency per Gray is known (Collins et al. 1996).

Given the results obtained with the enzymes, the authors concluded that the use of enzymes in the comet assay certainly increases its sensitivity, increasing the scope of the assay in fields such as genotoxicity testing and human biomonitoring.

In 2004, it was shown that Fpg also recognizes alkylating damage in DNA, particularly ring-opened N7 guanine adducts (N-7 alkylguanines) (Speit et al., 2004). These lesions, on alkaline treatment, are converted into ring-opened guanines, which are among the DNA alterations detected by Fpg. The lysis step of the comet assay, which is performed at pH 10 and occurs before the enzyme incubation, may be responsible for such conversion during the performance of the assay. Hansen et al. (2018) and, few year later, Muruzabal et al. (2020) demonstrated that Fpg-sensitive sites were not detected in cells treated with methyl methanesulfonate (MMS), an alkylating agent, if the lysis was performed at pH 7 but they were detected if the lysis was performed at pH 10.

4.1. Human biomonitoring

A field in which Fpg has been widely used is human biomonitoring; see the recent review by Azqueta et al. (2020). Recently, Møller et al. (2019) studied the effect of habitual consumption of fish, vegetables, fruits, salads, whole-grain bread and potatoes on the levels of oxidatively damaged DNA in a cross-sectional study. They found an inverse association between ingestion of fish and levels of Fpg-sensitive sites in women (after adjustment for various other lifestyle factors). Shaposhnikov et al. (2018) studied the potential protection by coffee consumption against effects of reactive oxygen in healthy volunteers, measuring the oxidation level of DNA in PBMCs by the enzyme-modified comet assay. The authors detected no effect on DNA damage, implying neither beneficial nor deleterious effects of coffee on human health.

Apart from nutritional studies, the Fpg-modified assay has been employed in physiological studies and investigations of occupational and other types of exposure. For instance, a positive association was found between age and DNA oxidation, in terms of Fpg-sensitive sites in blood cells (Humphreys et al., 2007; Mota et al., 2010). Cavallo et al. (2006) found a significantly higher level of Fpg-sites in blood and buccal cells from 'on the ground' airport workers compared with office workers at the same airport, and Løhr et al. (2015) showed a significant positive association between alcohol intake and Fpg-sensitive sites in men (but not in women). Williamson et al. (2020) showed higher levels of oxidative DNA damage (in terms of Fpg-sensitive sites) in healthy male participants after high-intensity exercise trials in hypoxia compared to normoxia.

The Fpg-modifed comet assay has also been used in the study of diseases; as an example, Biancini et al. (2015) compared basal DNA breaks and oxidation damage in Fabry disease patients and healthy controls. They found higher levels of Fpg-sites in the Fabry disease

patients.

4.2. Genotoxicity testing (in vitro and in vivo)

Another scientific discipline in which the Fpg-modified comet assay has been extensively used is genotoxicity testing. Indeed, several attempts to validate the *in vitro* Fpg-modified comet assay for genotoxicity testing have been performed. Smith et al. (2006) compared Fpg with two other enzymes (EndoIII and human 8-oxoguanine DNA-glycosylase 1 - hOGG1) for the ability to increase the sensitivity of the comet assay. For this purpose, mouse lymphoma cells (L5178Y) were treated with oxidizing and alkylating agents. All enzymes resulted in increases in the sensitivity of the assay; as is explained in the corresponding section, hOGG1 was the most specific for DNA oxidation lesions.

In 2013, Azqueta and co-workers evaluated the genotoxicity of selected known genotoxic compounds (with different mechanisms of action), non-genotoxic but cytotoxic chemicals and non-genotoxic non-cytotoxic chemicals in TK-6 cells using the Fpg-modified comet assay (Azqueta et al., 2013a). Genotoxic agents were detected at relevant concentrations without false positives, while non-genotoxic compounds gave no positive results. Thus, it was concluded that Fpg in combination with the comet assay increases dramatically its sensitivity without reducing its specificity.

Soloneski et al. (2017) studied the genotoxicity of a herbicide (imazethapyr) in mammalian CHO–K1 cells employing the enzyme-modified assay (with both EndoIII and Fpg). They concluded that DNA oxidation damage may be the underlying mechanism explaining the genotoxicity of the herbicide. In a more recent study, Meng et al. (2020) developed a co-culture of mouse primary hepatocytes and splenocytes as a model to evaluate genotoxicity *in vitro* based in the Fpg-modified comet assay. This model showed high sensitivity, discriminating between different known genotoxic compounds and non-genotoxic compounds.

Interference of Fpg with NPs, when using the Fpg-modified comet assay, has been described (Kain et al., 2012). However, the authors mixed Fpg with NPs, which create a protein corona effect that affects the enzyme activity, and other authors have claimed that such interference is unlikely when applying the assay correctly (Magdolenova et al., 2012). El Yamani et al., studied the genotoxic effects of titanium dioxide, zinc oxide, cerium oxide and silver nanomaterial (NMs) using the Fpg-modified comet assay in two different cell lines after short and long periods of treatment (i.e., 3 and 24 h) (El Yamani et al., 2017). All NMs were genotoxic and the inclusion of Fpg was crucial for the detection of the oxidized bases induced under some of the conditions tested. Iglesias et al. (2017a) studied the *in vitro* genotoxicity of several surface-modified poly(anhydride) NPs designed for oral drug delivery in human colon cell lines. They showed no effect on Fpg-sensitive sites.

Similarly, the Fpg-modified comet assay has also been applied for *in vivo* genotoxicity testing, especially to evaluate the mechanisms of action of carcinogenic chemicals. For instance, Ding et al. (2012) investigated the mechanisms of furan carcinogenicity in male F344 rats using the *in vivo* comet assay in combination with Fpg. Results showed a near-linear dose-response of oxidized purines at cancer bioassay doses. This allowed them to suggest a carcinogenic mode of action involving a secondary genotoxic mechanism associated with oxidation damage. Recently the Fpg-modified comet assay has been successfully applied to frozen liver, kidney and lung from untreated and MMS-treated rats (Azqueta et al., 2019a).

Genotoxicity assessment of NPs has also been carried out *in vivo*. Asare et al. (2016) determined the genotoxicity of TiO_2 and different sizes of silver NPs in liver, lung and testis from mice. Using the Fpg-modified comet assay, DNA oxidation lesions were compared in wild type and ogg1-knockout mice. Silver NPs induced Fpg-sensitive sites in testis and lung of WT mice while TiO_2 NPs did so only in testis. Ogg1-deficient mice presented higher levels of Fpg-sensitive sites in all organs, especially in liver, compared to the WT. Iglesias et al. (2017b) found an increase of Fpg-sensitive sites in the duodenum of mice exposed to 2000 mg/kg b.w. Of poly(anhydride) NPs. This effect was not observed at lower doses or in other organs of the gastrointestinal tract. A more recent publication showed the effects of aluminium and aluminium oxide NMs in different rat tissues after oral exposure (Jalili et al., 2020). There was an increase in Fpg-sensitive sites in bone marrow of rats administered with aluminium oxide NMs whereas aluminium NMs induced only a slight increase in blood cells.

4.3. Environmental biomonitoring and ecotoxicology

Fpg has also been used in combination with the comet assay to investigate the effects of chemicals and residues in the environment on the genetic material of organisms. As an example, Zhao et al. (2015) studied the DNA damage induced by a pesticide (monocrotophos) in peripheral erythrocytes of goldfish (*Carassius auratus*). After incubation with Fpg and EndoIII, the comet assay showed high levels of oxidized bases in DNA of erythrocytes of exposed fish in comparison to controls. Likewise, Pellegri et al. (2020) standardized a protocol for the comet assay in combination with Fpg for biomonitoring freshwater environments. Once standardized, the protocol was successfully applied, showing that the test organism *Daphnia magna*, combined with the Fpg-modified comet assay, was highly effective and sensitive at highlighting the presence of contaminants causing oxidative stress.

4.4. Other studies

The Fpg-modified comet assay has been applied to study the potential DNA protection capacity of several compounds (mainly antioxidants) and their effect on the DNA repair capacity of cells. Lorenzo et al. (2009) evaluated the protective effect of β-cryptoxanthin, a common carotenoid, on the DNA damage induce by a photosensitizer plus light (used to induce oxidized purines, mainly 8-oxoguanines), in HeLa and Caco-2 cells, by using the Fpg-modified comet assay. They showed that this compound protected against oxidation of DNA in both cell lines. They also studied the effect of β-cryptoxanthin on the removal of oxidized purines by performing the Fpg-modified comet assay at different times after the induction of the DNA lesions. Results showed that this carotenoid increased the DNA repair rate of the oxidized purines. Using a similar approach, Azqueta et al. (2013b) showed that vitamin C was not able to protect the DNA of HeLa cells from the damage induced by the photosensitizer plus light, and did not have any effect on the rate of DNA repair of the induced lesions. Recently, Huarte et al. (2020) evaluated the antioxidant effect of (poly)phenols from gastrointestinal-digested green pepper and from cooked (grilled) green peppers in colon HT-29 cells. Results indicated that green pepper (poly) phenols from both sources did not show genoprotection against oxidatively generated damage in HT-29 cells, but induced a slight pro-oxidant effect in terms of Fpg-sensitive sites.

5. uvrABC

The enzyme uvrABC is a bacterial ATP-dependent excision endonuclease comprising three subunits: uvrA, uvrB and uvrC. This protein complex is responsible for NER in prokaryotes, with a wide range of substrates, including UV-induced cyclobutane pyrimidine dimers and 6-4 photoproducts as well as bulky and DNA-distorting adducts (Sancar and Sancar, 1988). Briefly, the repair pathway is initiated when uvrA and uvrB form a complex with the DNA lesion. Subsequently, processing and incision are carried out by uvrC, thereby excising the oligonucleotide containing the lesion (Jia et al., 2009).

More recently, the repertoire of known substrates has increased to include other structurally and chemically different lesions, such as protein-DNA cross-links, oxidized bases, interstrand cross-links, tandem base damage and the presence of ribonucleotides in DNA (reviewed in Van Houten and Kad, 2014).

The enzyme was first used in combination with the comet assay in 1996 (Dušinská and Collins, 1996). The evaluation of uvrABC performance in the assay was performed with UV-irradiated HeLa cells and lymphocytes. However, according to the authors, uvrABC detected only a small fraction of the available lesions.

6. T4 endonuclease V (T4endoV)

The enzyme T4endoV is a DNA glycosylase specific for pyrimidine dimers (Gallagher and Duker, 1986). It was originally isolated from *E. coli* infected with T4 bacteriophage. The enzyme recognizes cyclobutane pyrimidine dimers (CPDs), induced by UV radiation (environmentally, mainly UVB) and consisting of two adjacent pyrimidines (usually thymines), covalently joined by a cyclobutane ring. T4endoV cleaves the glycosyl bond of the 5′-pyrimidine of the CPD, but also possesses an AP-lyase activity, so it subsequently breaks the phosphodiester bond 3′ of the glycosylase-generated abasic site (Schrock and Lloyd, 1993). Due to its AP-lyase activity, the enzyme is able to detect and cleave AP-sites, although with less efficiency than CPDs. Indeed, it was found that only about 60% of T4endoV-sensitive sites, commonly counted as CPDs, were true CPDs; the other 40% were AP-sites (Jiang et al., 2009).

Dizdaroglu et al. (1996) reported that, in addition to its well-known activity for CPDs, T4endoV also excises FapyAde from DNA through N-glycosylase activity, but estimated that the release was at a very low level in comparison to CPD excision (1–3% of that for CPDs). They employed gas chromatography/mass spectrometry (GC/MS), but under their conditions numerous other modified bases that were present along with FapyAde in DNA were not recognized by the enzyme.

The enzyme was used in combination with the comet assay for the first time to characterize rodent UV-sensitive mutant cell lines (Collins et al., 1997). In this work, the authors examined the responses of several cell lines to UV irradiation, monitoring the removal of CPDs with the T4endoV-modified comet assay. Thus, they were able to compare the rates of CPD excision among cell lines.

The enzyme T4endoV has been used in combination with the comet assay in different fields, mainly for the detection of CPDs after UV irradiation.

6.1. Human biomonitoring

The T4endoV-modified alkaline comet assay was applied to epithelial cells from the human lens capsule (Osnes-Ringen et al., 2013). Samples were obtained from patients undergoing cataract removal and analysed soon after being obtained and after 1 week in culture medium. The objective was to measure the basal DNA damage in this tissue but also to study possible cellular changes under *ex vivo* conditions. Low levels of T4endoV-sensitives sites were observed in all conditions tested. Fpg- and EndoIII-sensitives sites were also determined.

Using a similar approach, the T4endoV-modified comet assay was applied to the epithelium of corneas (Haug et al., 2013). The objective was to examine the effect of different storage conditions on corneas destined for transplantation; 10 samples were stored under hypothermic conditions in Optisol GS and then transferred to an organ culture medium at 32 °C for 1 week. There was a slight increase in the low levels of T4endoV-sensitives sites after 1 week in organ culture medium. Fpg and EndoIII were also used in this study. In a similar study but with a lower number of samples, the levels of T4endoV-sensitives sites were quite similar before and after the incubation (Azqueta et al., 2018).

6.2. Genotoxicity testing (in vitro and in vivo)

Regarding genotoxicity evaluation, several *in vitro* studies have been performed using human cells, either primary cultured or stable cell lines. For instance, Woollons et al. (1997, 1999) evaluated the genotoxic potential of artificial tanning lamps and sunbeds. Using T4endoV with the comet assay, the authors were able to detect the induction of CPDs in cultured human fibroblasts. Sparrow et al. (2003) studied the potential genotoxicity of the interaction between pyridinium bisretinoid A2E and blue light in retinal pigment epithelium cells. The comet assay was performed in combination with T4endoV, Fpg and EndoIII; Fpg- and EndoIII-sensitive sites were found, but no T4EndoV-sensitive sites, confirming that there was no direct influence of UV on DNA.

6.3. Environmental biomonitoring and ecotoxicology

T4endoV has also been applied in combination with the comet assay to evaluate UV radiation effects on plants. Sastre et al. (2001) evaluated the sensitivity of the enzyme-modified comet assay to detect UV-induced damage in *Rhodomonas* sp. DNA, and recommended the assay, including the enzyme-digestion step, as a suitable method to measure UV radiation-induced DNA damage in microalgae. They stressed the advantages of the technique (results obtained from individual unicellular microalgae; number of cells required; simple compared to other methods; relatively inexpensive). More recently, Holá et al. (2015) applied T4endoV in combination with the comet assay to evaluate responses to UVB radiation in plants. In particular, it was employed for the specific detection of CPDs and to evaluate the kinetics of their removal in the moss *Physcomitrella patens*.

6.4. Other studies

Sauvaigo et al. (1998) used the enzyme in combination with the comet assay to validate a method in which electrophoresed samples were also analysed using an indirect immunofluorescence detection with monoclonal antibodies. Human fibroblasts were irradiated with UVB and evaluated using the T4endoV-modified comet assay and the modified immunodetection assay. The use of the enzyme confirmed the specificity of the immunodetection approach; when T4endoV was used, lesions were digested thereby making them inaccessible for antibody detection, whereas the antibody response was linear along with the UVB light dose when there was no enzyme-digestion step.

Rafferty et al. (2003), Decome et al. (2005) and Robinson et al. (2010) employed the T4endoV-modified comet assay to study effects of different reagents on CPD levels in UV-irradiated cells (i.e. repair or prevention); selenium compounds, photolyase and exogenous photosensitizers were studied.

7. Uracil DNA glycosylase (Udg)

Udg is a ubiquitous BER enzyme specific for uracil present in DNA. Udg from *E. coli* was the first DNA glycosylase to be discovered and then it was shown that it is highly conserved in other bacteria, yeast, green plants, animals and even in mitochondria (Lindahl, 1974).

Udg detects and removes uracil present in both single- and double-stranded DNA, but not in RNA (Kow, 2002). The presence of uracil residues in DNA results from spontaneous deamination of cytosine (generating U:G mismatches) or as a result of misincorporation of dUMP opposite adenine during replication (generating U:A pairs). Specifically, Udg catalyzes the hydrolytic cleavage of the N-glycosidic bond between the sugar phosphate backbone and the uracil residue. Unlike other glycosylases (e.g. hOGG1, Fpg), Udg is not able to cleave the phosphodiester backbone of the generated abasic site, as it does not possess AP-lyase activity. Udg is also able to recognize less efficiently other lesions, such as 5-fluorouracil and deamination products (e.g. 5-OH-uracil and 5,6-dihydroxyuracil) (Zastawny et al., 1995; Kow, 2002).

Duthie and McMillan (1997) developed the Udg-modified version of the comet assay to detect misincorporated uracil in human DNA. The assay was carried out *in vitro* using human lymphocytes from healthy males and HeLa cells cultured in normal and folate-deficient medium, showing an increase in Udg-sensitive sites in cells grown in the folate-deficient medium. The authors confirmed the specificity of the modified protocol for the detection of misincorporated uracil in human

cells.

Duthie and collaborators used the Udg-modified comet assay in relation to folate status in several *in vitro* (Duthie and Hawdon, 1998; Duthie et al., 2000a, 2008), *in vivo* (Duthie et al., 2000b, 2010) and human studies (Narayanan et al., 2004; Basten et al., 2006). They recently published a review of applications of the Udg-modified comet assay to colon cancer (Catala et al., 2019), showing that a deficiency of folate induces an increase in uracil in the DNA and that repleting or increasing folate induces a corresponding decrease.

Human studies carried out by Duthie and collaborators applied the Udg-modified comet assay in blood cells (Narayanan et al., 2004; Basten et al., 2006); however, other groups have applied the assay to study the effect of folate status in target organs. Uracil levels were increased in colon biopsies from patients with polyps in comparison with control subjects (McGlynn et al., 2013), and a decrease was seen in a follow-up intervention study using folic acid (O'Reilly et al., 2016).

The Udg-comet assay has also been applied in research areas unrelated to folate status. Swain and Subba Rao (2011), applied the assay in isolated neurons and astrocytes from the cortex of young (7 days), adult (6 months) and old (2 years) rats and observed an increase in Udg-sensitive sites with age (along with SBs and hOGG1-sensitive sites).

The Udg-modified comet assay has also been applied to evaluate the level of basal and induced DNA damage in lymphocytes of children with acute lymphoblastic leukemia at different points of therapy with methotrexate (MTX), which causes misincorporation of uracil into DNA, and 6-mercaptopurine (6 MP) (Stanczyk et al., 2012). They observed that the level of Udg-sensitive sites increased in lymphocytes of patients after simultaneous treatment with 6 MP and MTX in comparison with the levels before treatment or 14 days after treatment, and with treatment with 6 MP alone. They did not observe differences between lymphocytes of patients and controls before the treatment.

8. Exonuclease III (ExoIII)

ExoIII, also known as AP endonuclease VI, is an AP endonuclease from *E. coli*. ExoIII catalyzes the hydrolysis of different bonds in double-stranded DNA and it is involved in repair processes (Demple and Harrison, 1994). It consists of a 3′-5′ exonuclease, releasing 5′ deoxynucleotides from the 3′ ends of DNA strands, an AP endonuclease, cleaving the phosphodiester backbone at AP-sites, and a DNA 3′-phosphatase (Rogers and Weiss, 1980).

The standard alkaline comet assay, in which the alkaline unwinding and the electrophoresis are performed at pH $>$ 13, already reveals AP-sites since they are converted into breaks under these alkaline conditions. That is maybe why this enzyme has only been used to a small extent in combination with the comet assay, notably in studies aimed at evaluating different pH-versions of the comet assay. ExoIII was first included in the comet assay protocol in 1999 by Angelis and colleagues. The authors were investigating various less sensitive versions of the comet assay, as in some human biomonitoring studies with subjects exposed to high levels of radiation or chemicals, basal levels of SBs were too high to measure accurately any additional enzyme-sensitive sites. The usefulness of an alkaline-neutral (A/N) comet assay (alkaline incubation followed by neutral electrophoresis) was evaluated in both animal and plant cells with different DNA-damaging agents and with a selection of lesion-specific enzymes. The ExoIII-modified comet assay was applied to *Vicia faba* root tip cells treated with various concentrations of either MMS or menadione to detect and compare the AP-sites detected with alkaline unwinding/neutral electrophoresis (A/N), alkaline unwinding/alkaline electrophoresis (A/A) and neutral pre-incubation/neutral electrophoresis (N/N). Results showed that some AP-sites are not detected if ExoIII is not used demonstrating that some AP-sites are resistant to A/N conditions and even to A/A conditions when the standard comet assay is used. (The A/A condition was the most sensitive version, due to the conversion of AP-sites into breaks, and the N/N condition gave a higher background in untreated cells that the authors attribute to a potential distortion of the nuclei due to the DNA not being denatured). The induction of SBs and AP-sites by alkylating mutagens following different treatment protocols (including an adaptation protocol, i.e., treatment with a low concentration followed by a postincubation and a treatment with a high concentration) was studied in meristematic nuclei of *Vicia faba* using A/N and A/A conditions (Angelis et al., 2000). ExoIII applied in the A/N comet assay induced extra breaks in all conditions tested indicating the presence of AP-sites after treatment with alkylating agents. When the adaptation protocol was applied, the level of ExoIII-sensitive sites decreased, showing that the repair of AP-sites contributes to the phenomenon of adaptation. This effect was not seen if protein synthesis was inhibited.

This enzyme has also been used in combination with the comet assay to characterize the origin of the SBs induced by the heterocyclic N-nitrosomorpholine (NMOR) in human colonic carcinoma Caco-2 cells and detected with the standard alkaline comet assay (Robichová and Slamenová 2001). NMOR induced a dose-dependent increase of DNA lesions when using the comet assay at pH $>$ 13 (unwinding and electrophoresis) while no lesions were detected at pH 12.1. A dose-dependent increase in ExoIII-sensitive sites was observed when the comet assay (unwinding and electrophoresis) was performed at pH12.1, indicating that NMOR induces AP-sites. With a similar objective, Rojas and collaborators employed ExoIII in combination with the comet assay in unstimulated and PHA-stimulated whole blood exposed to etoposide (Rojas et al., 2009). Non-stimulated blood cells showed a dose-dependent increase in DNA damage at pH $>$ 13 (unwinding and electrophoresis) while no damage was detected at 12.1. Meanwhile, stimulated blood cells showed a dose-dependent increase in DNA lesions under both conditions. A dose-dependent increase in ExoIII-sensitive sites was observed in non-stimulated blood cells after performing the comet assay (unwinding and electrophoresis) at pH 12.1. This increase was not observed in PHA-stimulated blood cells.

9. 3-Methyladenine DNA glycosylase II (AlkA)

AlkA is a monofunctional bacterial repair enzyme, with one of the broadest substrate ranges, being able to cleave both alkylated purines and pyrimidines (Krokan et al., 1997). Originally, AlkA was thought to be specifically involved in detecting and repairing alkylation damage, mainly 3-methyladenine; but it detects also 3-methylguanine, 7-methyladenine, 7-methylguanine, O^2-alkylcytosine, O^2-alkylthymine and hypoxanthine (Bjelland et al., 1994; Krokan et al., 1997; David and Williams, 1998). However, Berdal et al. (1998) showed that AlkA can also act non-specifically, removing normal base residues, with lower efficiency than for damaged bases but at biologically significant levels.

It was first used in combination with the comet assay by Collins et al. (2001). In this study, reaction conditions were optimized to detect alkylated bases, minimising non-specific reaction with normal bases. Thus, the enzyme was employed at different concentrations with a range of incubation times in untreated lymphocytes. Then, selected conditions were applied to human PBMCs treated with MMS, revealing high levels of DNA damage while low background levels were seen in normal cells. By employing a calibration based on X-irradiation of lymphocytes, it was calculated that the background level of alkylation damage detected with AlkA was about 0.8 sites per 10^9 Da of DNA, or 3000 alkylated bases per cell (though this might reflect the activity of AlkA on undamaged bases). This was very similar to the levels of oxidized base damage determined by the comet assay with Fpg and EndoIII.

AlkA has been used in a few *in vitro* genotoxicity studies, and also in occupational and clinical studies, by two research groups.

Human lymphocytes were used to study the genotoxic effects of nickel chloride (Woźniak and Błasiak, 2002), idarubicin and mitoxantrone (Błasiak et al. 2002), lead acetate (Woźniak and Błasiak, 2003), alloxan (Błasiak et al., 2003) and streptozotocin (Błasiak et al. 2004a). In the last case, HeLa cells were also used. Nickel chloride, idarubicin, lead acetate, alloxan and streptozotocin all induced AlkA-sensitive sites.

AlkA has also been employed to study the effect of asbestos and mineral fibres, in workers from a former asbestos cement plant and a rockwool factory respectively (Dušinská et al., 2004a, 2004b). Dušinská et al. (2004)a found a general positive association between the presence of alkylated bases in lymphocytes and age, and between alkylated bases and years of occupational exposure in workers exposed to asbestos. They did not find any effect of rockwool exposure on the levels of AlkA-sensitive sites (Dušinská et al., 2004b).

Błasiak et al. found no difference in the levels of alkylated lesions in lymphocytes between patients diagnosed with type 2 diabetes mellitus and controls (Błasiak et al., 2004b); nor were there differences in gastric mucosa cells from *Helicobacter pylori*-infected patients compared with non-infected controls (Arabski et al., 2005). This group also assessed the levels of alkylated bases in a case-control study on breast cancer patients and found that although there was no difference in AlkA-sensitive sites between breast cancer patients before chemotherapy and controls, the levels of these altered bases increased after chemotherapy (Błasiak et al., 2004c).

Fpg and Endo III were also employed in all the studies mentioned in this section, except in the study of Arabski et al. (2005), in which EndoIII was not used.

10. 8-Hydroxyguanine DNA-glycosylase (hOGG1)

hOGG1 is the eukaryotic counterpart of Fpg, first applied in the comet assay by Smith et al. (2006). hOGG1 is a DNA repair enzyme involved in base excision repair (BER), recognizing and catalyzing the removal of oxidized purines from double-stranded DNA (Boiteux and Radicella, 2000). hOGG1 possesses N-glycosylase and AP lyase activities, cleaving the N-glycosidic bond, releasing the damaged purine leaving an AP-site, and cleaving the phosphodiester bond on the 3' side of the AP-site (Lukina et al., 2013; Boiteux et al., 2017).

hOGG1 is specific for 8-oxo-7,8-dihydroguanine (8-oxoguanine), 8-oxoadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (David and Williams, 1998; Dizdaroglu, 2005; Lukina et al., 2013). 8-Oxoguanine has been used as a biomarker of oxidative stress and carcinogenicity and is considered the main consequences of oxidative damage to DNA (Angerer et al., 2007; Collins, 2009).

Smith et al. (2006) compared the substrate specificities of Fpg, EndoIII and hOGG1 by using compounds with different modes of action in mouse lymphoma cells. Thus, cells were treated with either MMS or ethylnitrosourea (ENU) to induce alkylation damage; and with potassium bromate to induce DNA oxidation damage (mainly 8-oxoguanine). After incubation with alkylating agents, Fpg and EndoIII produced significant increases in SBs; but there was no such increase with hOGG1. On the other hand, after potassium bromate treatment, similar large increases in both Fpg- and hOGG1-recognized break sites were induced, whereas the response with EndoIII was smaller. Overall, these results show that hOGG1 is more specific than Fpg or EndoIII for DNA oxidation damage (8-oxoguanines). Thus, after treatment with an agent of unknown mode of action, SBs induced by digestion with either Fpg or EndoIII cannot necessarily be ascribed to oxidation damage (Smith et al., 2006; Azqueta el at., 2013a; Hansen et al., 2018; Muruzabal et al., 2020). For this reason, hOGG1 appears to give more reliable estimates of DNA oxidation damage.

hOGG1 has been applied, *in vitro, in vivo* and in humans, to measure oxidation damage in various studies, though less extensively than Fpg or EndoIII.

10.1. Human biomonitoring

hOGG1 has not been much used in human biomonitoring though it has considerable potential. In a large human biomonitoring study, Lørh et al. (2015) studied the association between oxidized bases in PBMCs and metabolic risk factors in 1019 subjects from 18 to 93 years of age using the Fpg- and the hOGG1-modified comet assay (Lørh et al., 2015). They found an association between age and the levels of Fpg- and hOGG1-sensitive sites in women. hOGG1-sensitive sites were also associated with the plasma concentration of triglycerides.

In a more recent publication, Dinçer et al. (2019) hypothesized that polymorphisms in DNA repair genes may be related to alterations in the capacity for repair of DNA oxidation damage in Alzheimer's disease patients. Employing the hOGG1-modified comet assay, the authors showed that basal and oxidative damage to DNA was higher in Alzheimer's disease patients with Ser326Cys + Cys326Cys polymorphism compared to levels in patients with Ser326Cys polymorphism.

10.2. Genotoxicity testing (in vitro and in vivo)

Valdiglesias et al. (2011) evaluated DNA oxidation damage induced by the marine toxin okadaic acid (OA) in: human peripheral blood leukocytes, SHSY5Y cells (human neuroblastoma cell line) and HepG2 cells (human hepatocellular carcinoma cell line). To evaluate the possible induction of DNA oxidation damage, and based on the specificity conclusions of Smith et al. (2006), they used hOGG1 to measure 8-oxoguanine levels. This determination was made both in the presence and absence of S9 to assess whether OA acts directly or needs metabolic activation. Overall, the authors were able to detect OA-induced DNA oxidation damage directly in leukocytes, directly and indirectly in SHSY5Y cells, while it did not induce DNA damage in HepG2 cells. More recently, the hOGG1-modified comet assay was also applied *in vitro* to study the effects of Mycoplasma infection in cultured cells (Ji et al., 2019). In particular, the authors reported an induction of oxidation damage, in terms of hOGG1-sensitive sites, in cells infected with Mycoplasma as well as a decrease in repair capacity compared with non-infected cells.

Valdiglesias et al. (2012) evaluated *in vivo* the potential genotoxic effects of a fuel oil with characteristics similar to the oil spilled by the *Prestige* tanker. Wistar Han rats and Brown Norway rats were exposed to the fuel oil for 3 weeks using an inhalation chamber and genotoxicity was evaluated in leukocytes 72h and 15 days after the last exposure. To determine primary DNA damage, the standard comet assay version was employed while DNA oxidation damage was evaluated using the hOGG1-modified comet assay. Oil inhalation caused base oxidation in both rat strains, especially after 15 days of exposure, which according to the authors may be explained by the storage and later release of oil compounds. A recent publication also showed the application of the hOGG1-modified comet assay *in vivo*. Rašić et al. (2020) studied the mechanisms of toxicity of sterigmatocystin in male Wistar rats. Results after short-oral treatments showed that sterigmatocystin induced oxidative damage (in terms of hOGG1-sensitive sites) in liver and kidneys.

hOGG1 has also been applied for the genotoxicity assessment of NPs. For instance, Fernandez-Bertolez et al. (2019) evaluated the oxidative damage induced by iron oxide NPs on different nervous system cells (human SH-SY5Y neuronal and A172 glial cells). Their results indicated that induction of DNA oxidation damage was found after treatment of neuronal and glial cells with iron oxide NPs.

Pfuhler et al. (2017) assessed *in vivo* the potential DNA damage induced by 15-nm silica NPs. The standard and hOGG1-modified comet assays were applied to blood, kidney and liver samples from Wistar rats treated intravenously with silica NPs. Two positive control compounds, ethyl mehtanesulphonate (EMS) and potassium bromate, were administered orally to test for the proper functioning of the comet assay protocol. The use of the enzyme in combination with the comet assay sharply increased measurable DNA damage in animals treated with the high dose of NPs. Similarly, potassium bromate showed a strong DNA damage response with the hOGG1 protocol, especially in the kidney (the target organ of its carcinogenic activity), whereas no response was observed using the standard protocol. EMS led to a strong DNA response without addition of hOGG1, but the enzyme further boosted the DNA damage level, as the compound acts as a direct acting genotoxin but can

also induce oxidative stress.

11. Endonuclease IV (EndoIV)

The enzyme EndoIV, also known as deoxyribonuclease IV, is a bacterial AP endonuclease, discovered in *E. coli*. EndoIV has 3′ phosphodiesterase activity, cleaving the DNA backbone at the AP-site to produce a SB with 3' OH termini (Jilani et al., 2003; Daley et al., 2010).

As explained before, AP-sites are already detected with the alkaline comet assay since they are transformed into breaks in alkaline conditions. That is one reason why this enzyme has not been extensively used with the comet assay. The enzyme was applied in a modified neutral comet assay (preincubation and electrophoresis at pH 8.5) by Holt and Georgakilas (2007), along with Fpg and EndoIII, to evaluate the possible accumulation of DSBs and oxidized DNA bases in leukemia cells exposed to gamma irradiation at equivalent doses to those employed in radiotherapy. EndoIV was used since it detects AP-sites including those caused by oxidation, as well as urea residues in DNA. A dose-dependent increase in enzyme-sensitives sites was detected with all three enzymes. (NOTE: The neutral comet assay not only detects DSBs but also SSBs; Collins et al., 2008; Azqueta and Collins 2013).

There appears to be only other article describing the use of EndoIV in combination with the comet assay. Gordon-Thomson et al. (2012) investigated whether the active vitamin D hormone (1α, 25 dihydroxyvitamin D_3) protects human keratinocytes from UV-induced DNA damage and whether reactive nitrogen species (RNS) may be involved in its production. For this purpose, the enzyme-modified comet assay, with alkaline unwinding and electrophoresis at pH 12.1, was employed with EndoIV, T4endoV and hOGG1 for the detection of AP-sites, cyclobutane pyrimidine dimers (CPD) and AP-sites, and 8-oxoG respectively. EndoIV-, T4endoV- and hOGG1-sensitive sites were detected in UV-irradiated human keratinocytes, but they were reduced by vitamin D hormone. EndoIV was employed to discriminate between CPD and AP-sites; the putative CPD fraction was calculated by subtracting the net EndoIV-sensitive sites from the T4endoV sensitive sites.

12. Endonuclease III-like protein 1 (NTH1)

NTH1 is the eukaryotic counterpart of bacterial EndoIII and, like its bacterial homologue, possesses N-glycosylase activity for the excision of the damaged DNA base, leaving an AP-site, and AP-lyase for its subsequent cleavage (Aspinwall et al., 1997). Regarding NTH1 specificity, the enzyme excises oxidized pyrimidine residues such as 5-OH-cytosine, thymine glycol, 5-OH-6-hydrotymine, 5,6-dihydroxycytosine and 5-hydroxyuracil, as well as Fapy residues (Dizdaroglu et al., 1999; Luna et al., 2000). Indeed, Kafuchi and colleagues (2004) compared the excision levels of NTH1 and EndoIII towards different lesions and showed that, unlinke EndoIII, NTH1 excises Fapy-guanine residues (Kafuchi et al., 2004).

NTH1 was used for the first time in combination with the comet assay by Morawiec et al. (2008) to determine the level of endogenous DNA damage in lymphocytes of children with Down syndrome and controls. The authors studied endogenous oxidative DNA damage with the NTH1- and Fpg-modified comet assay. Results showed that basal oxidative DNA, in terms of both NTH1- and Fpg-sensitive sites, was higher in Down syndrome children than in controls.

According to our knowledge, NTH1 was used with the comet assay once more, in a study performed by the same group (Szaflik et al., 2009) to determine endogenous oxidative DNA damage in lymphocytes of patients with age-related macular degeneration (AMD) and in age-matched healthy individuals using the comet assay combined with NTH1 and Fpg. Lymphocytes from AMD patients showed higher levels of NTH1- and Fpg-sensitive compared with healthy controls.

13. Methyladenine DNA glycosylase (AlkD)

AlkD is a monofunctional repair enzyme (from the soil bacterium *Bacillus cereus*) that recognizes and incises alkylated bases in DNA. It is involved in BER of N-alkylated purine products and is specific for 3-methyladenine and 7-methylguanine showing no activity towards other important base lesions such as deaminated adenine (hypoxanthine), 1,N^6-ethenoadenine or 8-oxoguanines (Alseth et al., 2006; Hašplová et al. 2012).

Hašplová et al. (2012) published the first report on the use of AlkD with the comet assay. They optimized conditions for the AlkD-modified comet and the ability of AlkD to detect alkylated bases was evaluated *in vitro* using human lymphoblastoid (TK-6) cells. The background level of alkylation damage in TK-6 cells was at most 10% of DNA in tail, corresponding to about 0.3 sites per 10^9 Da of DNA or roughly 1000 alkylated bases per cell.

According to our knowledge, in addition to the paper by Hašplová et al. (2012), only one other study, by Ramos et al. (2013), has been reported. The potential of water extracts from different *Hypericum* species and some of their main phenolic compounds to prevent and repair oxidation and alkylation damage in colon cells was investigated, by treating HT29 cells with H_2O_2 and MMS respectively. Both Fpg and AlkD were employed; after MMS treatment, both enzymes recognized DNA damage but the levels detected by Fpg were higher than those by AlkD, probably reflecting the different specificities of the enzymes.

14. Alkyladenine DNA glycosylase (hAAG)

hAAG recognizes and incises alkylated bases. Specifically, it detects 3-methyladenine and 7-methylguanine (O'Connor, 1993) though there is a recent report that it also detects 1-methylguanine and has activity toward ethenoadenines and hypoxanthine (Lee et al., 2009). hAAG is a monofunctional glycosylase that releases the N-alkyl-adduct from DNA leaving an abasic site (Lau et al., 1998) - converted to a break under the alkaline conditions of the comet assay.

Very recently, Muruzabal et al. (2020) published the first paper in which hAAG was used in combination with the comet assay. They titrated the enzyme using untreated and MMS-treated TK-6 cells as substrate. hAAG was able to detect the alkylated bases induced by MMS in a dose-dependent manner but, as expected, it was not able to detect oxidized lesions induced by potassium bromate. They also compared the abilities of Fpg and hAAG to detect alkylated bases when combined with the comet assay; Fpg detects the ring-opened purines derived from some alkylated lesions by exposure of nucleoids to the mildly alkaline conditions of lysis, while hAAG detects the alkylated bases present in the nucleoids (as well as the ring-opened purines).

This new enzyme can be very useful, for the detection not only of alkylated bases but also of ethenoadenines and hypoxanthine. In contrast to AlKA and AlkD, hAAG is currently commercially available, which may favour its use.

15. General discussion and future of the enzyme-modified alkaline comet assay

The enzyme-modified comet assay was first applied in a study of antioxidant protection in humans, and human biomonitoring remains one of the most common areas in which it is used. Generally, the aim is to measure the level of DNA oxidation damage, and so it is not surprising that the enzymes used most frequently are Fpg and EndoIII, detecting oxidized purines and pyrimidines respectively. The fact that Fpg also detects alkylated bases is a complication; however, hOGG1 has the advantage of greater specificity, and indeed is catching up on Fpg after a late start.

Other enzymes have specific uses in the human biomonitoring field; for instance, the use of Udg has shown that folate supplementation decreases the level of uracil misincorporation into DNA (Basten et al., 2006),

and T4endoV was applied to lens epithelial tissue from cataract patients (Osnes-Ringen et al., 2012). But on the whole, little use has been made of enzymes apart from those recognizing oxidized bases.

Very recently a review on the use of the comet assay in human biomonitoring studies using both the standard and the enzyme-modified comet assay was published (Azqueta et al., 2020). This work describes the effect of different factors on the level of DNA damage and highlights the lack of standardized procedures to collect and store specimens. Moreover, it gives some recommendations for statistical analysis.

The ability to detect DNA lesions other than SBs is of considerable value in genotoxicity testing, if the aim is (as it should be) to avoid false negative results as much as possible. Fpg, as already mentioned, detects (some) alkylated bases, and it greatly increases the sensitivity of the comet assay in detecting the effect of MMS, but also the indirect effects of some non-alkylating agents, such as bulky adduct-inducing benzo(a) pyrene and 4-nitroquinoline-1-oxide (4NQO) (Azqueta et al. 2013). (Unfortunately, we do not have an enzyme that can directly detect bulky adducts; uvrABC has not given satisfactory results in this assay so far.)

Fpg is in fact quite widely used in genotoxicity testing, both *in vitro* and *in vivo*, with the aim of detecting oxidation damage. The potential for detection of a range of lesions (alkylated bases, ethenoadenines and hypoxanthine) with hAAG has recently been demonstrated (Muruzabal et al., 2020). The usefulness of the enzyme-linked comet assay in genotoxicity testing is generally recognized, to judge by the fact that Fpg and EndoIII have been employed in more studies in this than in any other field. It is therefore somewhat surprising that there is not an OECD test guideline for the *in vitro* comet assay (including the use of enzymes). Regarding the *in vivo* comet assay, the existing OECD test guideline (OECD, 2016) does not include the use of enzymes. A recent article by some of the current authors reviews publications in which enzymes have been employed *in vivo* and makes the case for an extension of the OECD guideline to include the enzyme modification (Collins et al., 2020).

As discussed in the previous paragraphs, the enzyme-modified comet assay increases the sensitivity of the standard version in detecting genotoxic compounds since it is able to detect genotoxic compounds that induce other DNA lesions apart from SBs and ALS. Moreover, it also increases the sensitivity of the standard assay in terms of the concentration of a genotoxin at which the genotoxic effect is detected; this has been observed both *in vitro* (e.g., Smith 2006; Azqueta et al. 2013) and *in vivo* (e.g. Azqueta et al. 2019; Collins et al., 2020). This fact can overcome the potential false positive results obtained at cytotoxic concentrations when the standard assay is used.

Enzymes are useful to investigate the mechanism of action of a given chemical known to be cytotoxic/genotoxic; applying a battery of enzymes to cell samples taken at different times during or after the treatment can determine whether the chemical causes direct or indirect oxidation damage, or alkylation, or of course SBs, and can give information about the cellular response - whether and how quickly the damage is repaired. The enzyme-modified comet assay is in fact recommended by some agencies as an 'indicator assay' to be used where an equivocal or positive result is obtained, to elucidate the mode of action (EFSA, 2017).

The enzyme-modified comet assay can be also performed in a medium or a high-throughput way using the 12 minigels/slide format (Shaposhnikov et al., 2010), GelBond films with different numbers of minigels (following the standard 24, 48 or 96 well formats) (Gutzkow et al., 2013), or the CometChip technology (Ge et al., 2014). The great advantage of the 12 minigels/slide format is that, by employing the 12 gel chamber unit, each of the minigels can be incubated separately (with different enzymes, for example).

It is worth mentioning that the enzyme-modified comet assay has also been applied to evaluate DNA methylation. The restriction endonucleases *Hpa*II and *Msp*I recognize the same nucleotide sequence but with different methylation sensitivity. In 2010, they were used in combination with the comet assay to detect global methylation in individual cells (Wentzel et al., 2010). More recently, the enzyme McrBC, which converts 5-methylcytosine into DNA breaks, was successfully used for the same purpose using the CometChip technology (Townsend et al., 2017).

Most of the enzymes used with the comet assay are commercially available. Other enzymes, from plants, for example, might be of value, and would be worth testing in a research context.

It is important to titrate each batch to find the incubation conditions (concentration, time) that give optimal detection of lesions (Muruzabal et al., 2019). Too low a concentration or too short a time will mean missing some lesions; on the other hand, too high a concentration or too long a time may cause non-specific damage.

Sometimes two enzymes have been applied in combination; for instance, Fpg and EndoIII to detect the total DNA base oxidation - giving an increased sensitivity, but no information on the relative amounts of different lesions. In view of the lack of an enzyme to detect bulky adducts, a nuclear extract could be used, on the assumption that it will contain all relevant enzymes (Wang et al., 2005). This will increase sensitivity, but specificity will be low, as all kinds of lesions will be detected. There is also a danger of non-specific nucleases breaking the DNA.

16. Future perspectives

We can hope for:

- more enzymes, with varied specificities, used in an analytical way to define modes of action
- modification of the OECD *in vivo* test guideline to include the use of enzymes
- an OECD guideline for the *in vitro* comet assay, including the use of enzymes
- more applications of the enzyme-modified comet assay in the field of ecogenotoxicology

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 4

The enzyme-modified comet assay: Enzyme incubation step in 2- vs 12-gels/slide systems

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The enzyme-modified comet assay: Enzyme incubation step in 2 vs 12-gels/slide systems

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ABSTRACT

The enzyme-modified comet assay is a commonly used method to detect specific DNA lesions. However, still a lot of errors are made by many users, leading to dubious results and even misinterpretations. This technical note describes some critical points in the use of the enzyme-modified comet assay, such as the enzyme concentration, the time of incubation, the format used and the equipment. To illustrate the importance of these conditions/parameters, titration experiments of formamidopyrimidine DNA glycosylase (Fpg) were performed using the 2 gels/slide and the 12 minigels/slide formats (plus the 12-Gel Comet Assay Unit™). Incubation times of 15 and 30 min, and 1 h were used. Results showed that the 12 minigels/slide system requires a lower volume and concentration of Fpg. A longer time of incubation has a bigger impact when using such format.

Moreover, the paper describes how to perform and interpret a titration experiment when using the enzyme-modified comet assay.

1. Introduction

The enzyme-modified alkaline comet assay was developed in 1993 by Collins et al [1]. They used endonuclease III (endo III) to measure the oxidized bases in human lymphocytes. To set up the assay, HeLa cells treated with different concentrations of H_2O_2 and subsequently incubated at 37 °C to allow repair of the DNA strand breaks (SBs) were used as substrate. In this way, they demonstrated the suitability of this assay to measure oxidized bases (i.e., endoIII sensitive-sites).

Since then, several enzymes have been used in combination with the comet assay to measure different DNA lesions and even DNA methylation [2]. The use of the enzymes in combination with the comet assay represents a huge advantage in all the fields in which the comet assay is used; genotoxicity testing, human biomonitoring, ecogenotoxicology and basic research. It allows the detection of different DNA lesions apart from simple SBs detected by the standard alkaline comet assay. It is a great tool to study the various DNA lesions induced by different chemicals, particulate matter, and radiation.

The enzyme-modified comet assay protocol adds one step to the standard alkaline comet assay: the incubation of the nucleoids, obtained after the lysis step, with the enzyme. This step can be done using different approaches. When using 1, 2 or 3 'big' gels/slide, the incubation of the nucleoids is usually done by adding a drop of enzyme on top of the gel and covering it with a coverslip. In the case of 12 minigels/slide [3], a commercial metal chamber (12-Gel Comet Assay Unit™, NorGenoTech, Oslo, Norway) can be used to incubate each of the gels separately; in this case a certain volume is added in each well of the chamber which contains one minigel. Alternatively to these approaches, slides can be submerged in a bath, or a Coplin jar, containing the enzyme. GelBond™ films are used to accommodate 24, 48 or 96 gels/slide and the incubation with the enzyme can only be done by submerging them in a bath containing the enzyme [4]. Obviously, submerging the slides/GelBond film in a bath implies the use of a greater amount of enzyme than the other options. Nucleoids incubated with enzyme reaction buffer (simply called buffer in the rest of the manuscript) are used as control; the net Fpg-sensitive sites are calculated by subtracting the DNA damage in the nucleoids incubated with the buffer from the damage seen in the presence of the enzyme (damage being expressed in terms of the extent of migration of DNA into the comet tail).

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When adding the enzyme (or the buffer) to the gels within an experiment, slides are kept cold (e.g. placed on a cold metal plate). This avoids the activation of the enzyme before slides are transferred to 37 °C for optimal enzyme efficiency, and ensures that the same time of enzyme incubation obtains in all the gels/samples.

The incubation of the nucleoids with the enzyme or the buffer is most commonly performed in an incubator at 37 °C. Slides that are not incubated by submerging them in baths, are placed in moist boxes and then in the incubator. However, a microscope slide incubator ('slide moat') is also an option used by some groups to incubate the slides.

Among all the enzymes, formamidopyrimidine DNA glycosylase (Fpg) is the most used. It detects the oxidized purine 8-oxo-7,8-dihydroguanine (8-oxo-Gua), formamidopyrimidines (i.e., ring-opened adenine or guanine) and ring-opened N7 guanines adducts produced by alkylating agents [5–7]. This enzyme was first used in combination with the comet assay in 1996 [5]. To set up the assay, HeLa cells were treated with different concentrations of H_2O_2 (on ice) and subsequently incubated at 37 °C to allow the repair of the SBs. The assay was also applied to human lymphocytes. Nowadays the Fpg comet assay is extensively used in human biomonitoring and in genotoxicity testing at research level [8–10].

Since there are several options to incubate the nucleoids with the enzymes or the buffer, each option may require different conditions in terms of enzyme concentration and incubation time. So a titration experiment is recommended for each procedure. The optimal concentration elucidated from the titration experiments should detect the maximum enzyme sensitive-sites without inducing non-specific breaks. To do so, substrate nucleoids containing the correspondent lesions and substrate without lesions should be used.

In this technical paper, we describe the titrations of Fpg, using for comparison two gel formats and two approaches to the Fpg incubation. The formats and approaches used are: a) 2 gels/slide system in which gels were incubated with Fpg or buffer by adding a drop on the gel and placing a coverslip on top of it; and b) 12 minigels/slide in which gels were incubated with Fpg or buffer by using the 12-Gel Comet Assay Unit™ and adding a certain volume in each well. In both approaches three incubation times were studied: 15 and 30 min, and 1 h. Moreover, how to perform the titration of an enzyme will be described.

2. Material and methods

2.1. Cell culture

TK-6 cells (human-derived lymphoblastoid cell line) were obtained from the American Type Culture Collection (ATCC). They were grown in RPMI (Roswell Park Memorial Institute) medium containing D-glucose, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate (ref. A10491-01, Gibco) and supplemented with 10% heat-inactivated fetal calf serum, 100U/ml penicillin and 0.1 mg/ml streptomycin (all from Gibco). Cells were maintained as a suspension culture in continuous agitation at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Treatment and freezing of cells

TK-6 cells at 1×10^6 cells/ml in culture medium without fetal calf serum were treated with 1.25 mM $KBrO_3$ during 3 h in continuous agitation at 37 °C in a humidified atmosphere with 5% CO_2. The $KBrO_3$ concentration was chosen after performing preliminary concentration response studies; 1.25 mM $KBrO_3$ induces a high amount of oxidized purines without concurrent generation of SBs.

After the treatment, cells were centrifuged and washed twice with phosphate-buffered saline (PBS). Treated and non-treated cells were frozen at 1×10^6 cells/ml in 0.5 ml aliquots in culture medium containing 10% DMSO in cryotubes. Cells were frozen by using the freezing container Mr. Frosty (Thermo Scientific, Nalgene). The container including the cryotubes was placed in a freezer at -80 °C at least overnight. After that, the cryotubes were transferred to boxes and kept at -80 °C until the analysis. Cells were kept cold during the whole process to prevent DNA repair. Different cell batches were used.

2.3. Comet assay – fpg titration

The titration of Fpg was carried out using 2 gels/slide and the medium-throughput format of 12 minigels/slide [3]. Fpg was provided by NorGenoTech (Oslo, Norway); it is a crude extract from an overproducing strain of *Escherichia coli*. To titrate the enzymes nucleoids containing 8-oxoguaine in the DNA, from cells treated with $KBrO_3$ (see previous section) were used.

$KBrO_3$-treated and untreated cells were quickly thawed by immersing the cryotube in a water bath at 37 °C and washed in 10 ml of cold PBS by centrifugation. After that, cells were suspended in PBS at 1×10^6 cells/ml for the 2 gel/slide format and at 2.5×10^5 cells/ml for the 12 minigels/slide format. Thirty microliters of the cells suspension was mixed with 140 µl of 1% low melting point agarose in PBS at 37 °C. In the case of the 2 gels/slide format, 2 aliquots of 70 µl of the correspondent cell suspension were placed on agarose-precoated slides and a 20 x 20 mm coverslip was placed on top of each of them. After 2–3 min on a cold metal plate (placed on ice), the coverslips were removed. In the case of the 12 minigels/slide format, 12 aliquots of 5 µl each of the corresponding cell suspension were placed on agarose-precoated slides. Slides were placed on the bottom metal holder of the 12-Gel Comet Assay Unit™ (NorGenoTech, Oslo, Norway) which contains a template to set the minigels in certain positions (two rows of six). The metal plate was previously placed in the fridge for cooling so the gels are set instantaneously.

After the gels were prepared, slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 M Tris base, pH 10 and 1% Triton X-100) at 4 °C for 1 h.

Before the enzyme/buffer treatment, slides were washed three times, 5 min each at 4 °C, with the reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0). Meanwhile the different concentrations of Fpg to test were prepared by making serial dilutions using the buffer. Slides were then placed on a cold metal plate to add the enzyme or the buffer. In the case of the 2 gels/slide format, 50 µl of each concentration of enzyme or buffer, were added on each gel and a 22 x 22 mm coverslip was placed on top. In the case of the 12 minigels/slide format, slides were transferred to a cold 12-Gel Comet Assay Unit™ to incubate each gel in separate wells. Thirty microliters of Fpg or buffer were added to each well (and a clean slide was placed on top of the unit to cover all wells); each test concentration of Fpg was evaluated in duplicate using two minigels. Slides (2 gels/slide format) and the 12-Gel Comet Assay Unit™ (12 gels/slide format) were then transferred to a pre-heated moist box and placed in the incubator at 37 °C for 15 min, 30 min or 1 h.

After the incubation, slides/units were placed on a cold plate to stop the Fpg reaction. Then, coverslips (2 gels/slide format) were removed and the slides were taken out from the unit (12 minigels/slide). All the slides (i.e., 2 gels/slide and 12 minigels/slide) were transferred to the electrophoresis tank and incubated for 40 min at 4 °C in the electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) to allow unwinding. Then, electrophoresis was carried out at 1.2 V/cm for 20 min.

After the electrophoresis, slides were neutralized by washing them in PBS for 10 min and then in distilled water for 10 min. Drying the slides is crucial when the 12 minigels/slide is used to avoid the edge effect (i.e. comets going in different angles) [11]. Slides containing 2 gels were air dried at room temperature, while slides containing the 12 minigels were immersed in 70% ethanol for 15 min and in absolute ethanol for a further 15 min before letting them air dry at room temperature.

Comets were stained with a drop of 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) on top of each gel (drop of 35 µl for the big gels

and 5 µl for the minigels) and coverslips were used to cover them (22 x 22 mm to cover a big gel and 24 x 60 mm to cover all the minigels on a slide). Slides were incubated with DAPI at room temperature for at least 30 min before the analysis. The semi-automated image analysis system Comet Assay IV (Perceptive Instruments) was used to evaluate 50 comets per gel (100/condition). The percentage of DNA in tail was the descriptor used for each comet, and the median % tail DNA of 100 comets was taken as the measure of DNA damage for each condition.

Net Fpg-sensitive sites were calculated by subtracting the % tail DNA obtained after buffer incubation from that obtained after the Fpg incubation with the different concentrations.

Different strategies to incubate the minigels using the 12-Gel Comet Assay Unit™were explored. Nucleoids from $KBrO_3$-treated cells were incubated with different dilutions of Fpg for 15 min in three ways: 1) the unit was placed in a pre-heated moist box and the box transferred to the incubator at 37 °C (as performed in the above experiments, 2) the unit was transferred directly to the incubator at 37 °C, and 3) the unit was placed on the bench at room temperature. As explained before, the enzyme was added to the wells of the 12-Gel Comet Assay Unit™ in the cold.

2.4. Fpg concentrations used for the titration experiments

In preliminary studies a wide range of Fpg concentrations were used for each format and each time of incubation. Five dilutions from the crude extracts were tested from 1/1,000 to 1/10,000,000 using a dilution factor of 10. After the analysis of the results a narrower range of Fpg concentration was tested for each format. In the case of the 2 gels/slide format the dilution tested were: 1/10,000, 1/30,000, 1/100,000, 1/300,000 and 1/1,000,000, while in the case of the 12 minigels/slide format, the dilutions tested were 1/100,000, 1/300,000, 1/1,000,000, 1/3,000,000 and 1/10,000,000.

2.5. Statistics

Mean % of DNA in tail from two independent experiments were calculated. These values together with the individual experimental values (as error bars) are presented in Figs. 2 and 3. Fig. 1 shows the mean % of DNA in tail from duplicate gels within one representative experiment.

3. Results

Results obtained in the preliminary studies, in which several dilutions (i.e., from 1/1,000 to 1/10,000,000) using a dilution factor of 10 were tested, are not shown. These experiments were used to select the final range of dilutions used; from 1/10,000 to 1/1,000,000 when using the 2 gels/slide format and from 1/100,000 to 1/10,000,000 when using the 12 minigels/slide format. To illustrate the effect observed when using too high concentrations of enzyme, results obtained using 1/1,000 to 1/100,000 dilutions of Fpg during 1 h incubation with the 12 minigels/slide system are shown in Fig. 1. As can be seen, the use of high concentrations of enzyme produces breaks (i.e., non-specific nuclease activity) in untreated cells.

Fig. 2a–c show the titration curves of the Fpg using 2 gels/slide after 15 and 30 min, and 1 h of incubation. The highest concentration tested (i.e., 1/10,000 dilution) induced a clear increase in Fpg-sensitive sites after 1 h of incubation in untreated cells. Regarding the $KBrO_3$-treated cells, there is a clear concentration response after all times of incubation; a clear increase in the net Fpg-sensitive is observed when the gels were incubated for 30 min in comparison with 15 min. However, just a moderate increase was observed when the gels were incubated during 1 h in comparison with 30 min A plateau in terms of enzyme concentration was only reached when using 1 h of incubation. Taking into account these figures, the Fpg dilution and the time of incubation selected for future experiments with this crude extract and the 2 gels/slide format would be 1/30,000 and 1 h. Using these conditions a wide range of Fpg-sensitive sites can be detected; from 0 to approximately 75% DNA in tail.

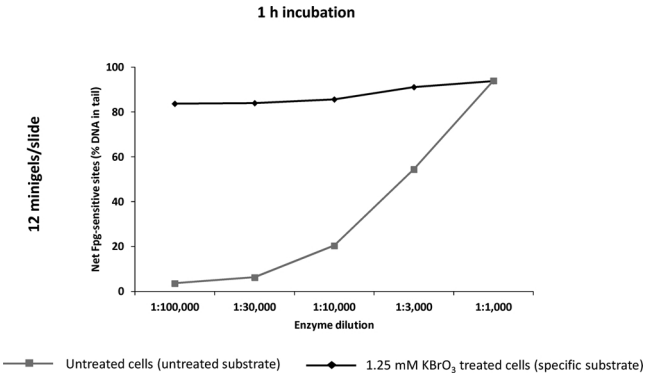

Fig. 1. Fpg titration experiment using 12 gels/slide format and 1 h of incubation. Net Fpg-sensitive sites are represented as % tail intensity. Black lines and diamonds represent the data obtained using 1.25 mM $KBrO_3$ treated cells as substrate while grey lines and square symbols represent the data obtained using untreated cells as substrate. Data represent mean values of duplicate gels from a representative experiment.

Fig. 2c, e and f show the titration curves of the Fpg using 12 minigels/slide after 15 and 30 min, and 1 h of incubation. None of the dilutions tested showed non-specific activity; they did not detect Fpg-sensitive sites in untreated cells (or very low levels). A clear concentration response was seen in cells treated with $KBrO_3$. In this case, a large increase in the net Fpg-sensitive sites was observed when the gels were incubated for 30 min in comparison with 15 min, and 1 h in comparison with 30 min. A plateau was only reached when using 1 h of incubation. In preliminary studies, 1.25 h incubation was also tested; results were similar to those obtained after 1 h of incubation (data not shown). The Fpg dilution and the time of incubation selected for future experiments using this crude extract and the 12 minigels/slide system (with the enzyme incubation in the metal chamber and inside a pre-heated moist box in an incubator) would be 1/300,000 and 1 h. Using these conditions a wide range of Fpg-sensitive sites can be detected; from 0 till approximately 75% DNA in tail.

As can be observed, the selected dilutions for further experiments are quite different depending on the comet assay format used (i.e., 2 gels/slide vs 12 minigels/slide). The 12 minigels/slide system requires lower Fpg concentration.

Fig. 3 shows the net Fpg-sensitive sites obtained after incubating the nucleoids from $KBrO_3$-treated cells with Fpg for 15 min using the 12-Gel Comet Assay Unit™ and different strategies. A dose concentration was observed in all cases. As can be observed, higher levels of net Fpg-sensitive sites were obtained when placing the 12-Gel Comet Assay Unit™ in a pre-heated moist box in an incubator at 37 °C, compared to the levels obtained after placing the unit directly in the incubator or leaving the unit on the bench at room temperature. Similar results were obtained when using the last two strategies.

4. Discussion

Differences in the comet assay protocols used by different research groups make it hard to compare results between laboratories. Relatively high inter-laboratory variation has been reported in various studies [12–16]. Moreover, quite high inter-experimental variation as well as intra-assay variation has also been reported [2,4,14]. In this regard, important factors influencing the outcome of the comet assay have been detected; final agarose concentration, duration of lysis, duration of alkaline treatment, duration and voltage applied during electrophoresis and scoring [17].

When using the comet assay in combination with enzymes, it is

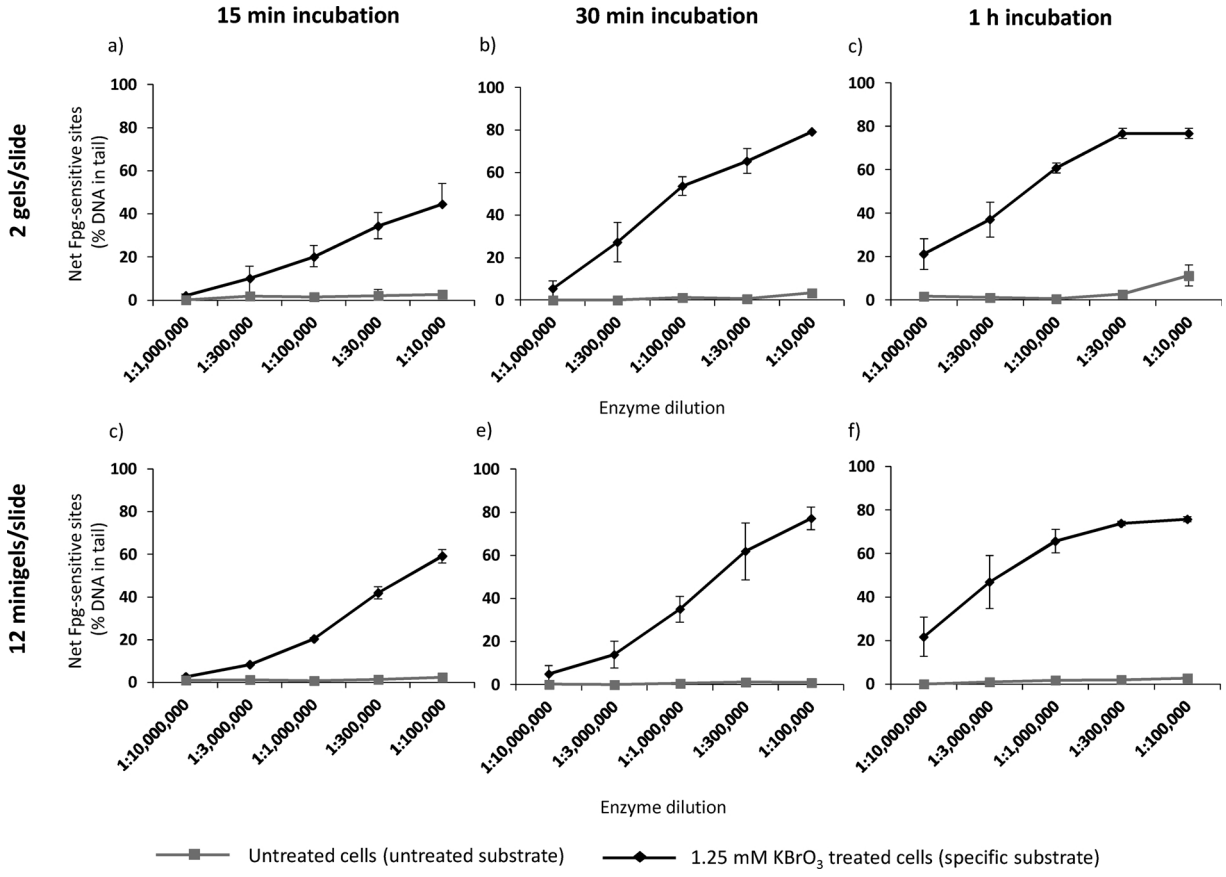

Fig. 2. Fpg titration experiments performed using the 2 gels/slide (a, b and c) and the 12 gels/slide (d, e and f) formats after 15 min (a and d), 30 min (b and e) or 1 h (c and f) of incubation. Net Fpg-sensitive sites are represented as % tail intensity. Black lines and diamonds represent the data obtained using 1.25 mM $KBrO_3$ treated cells as substrate while grey lines and square symbols represent the data obtained using untreated cells as substrate. Data are mean values from two experiments, and the bars indicate the range of values.

obvious that enzyme concentration and incubation time are of critical importance. The concentration should be high enough to detect the maximum amount of enzyme-sensitive lesions without producing non-specific breaks (see Fig. 1 as an example of non-specific breaks when using high concentrations of enzyme). To elucidate such concentrations, and the optimal time of incubation, titration experiments should be performed. As shown in this paper, one condition can be compensated by another; a lower concentration of enzyme can be compensated by a longer time of incubation. For instance, Fig. 2 shows that the Fpg dilution for future experiments (i.e., the selected concentration after the titration experiments) could be either 1/10,000 for 30 min of incubation or 1/30,000 for 1 h in the case of the 2 gels/slide format, and either 1/100,000 for 30 min or 1/300,000 for 1 h in the case of the 12 gels/slide. In these 4 cases 75% DNA in tail was detected without having unspecific activity. However, it is important to give the enzyme enough time to complete the reaction in order to obtain an accurate measure of the lesions present.

The non-specific activity shown in untreated nucleoids when using high concentration of enzymes could have two origins; they could be due to the presence of other nucleases, since we are working with a crude extract, but also to erroneous incision due to high concentration of enzyme and the lack of lesions. It is worth mentioning that pure commercial enzymes, which do not contain other nucleases, also present non-specific activity (data not shown). Enzyme concentration and time of incubation are obviously critical parameters; however, as can be seen in Fig. 2, the way the incubation step is performed is also critical. In this work, we have titrated a crude extract of Fpg using three times of incubation (i.e., 15 and 30 min, and 1 h) and two different formats. The formats used, 2 gels/slide (20 x 20 mm square gels) and 12 minigels/slide (5 µl dome shaped gels), use a different approach to carry out the

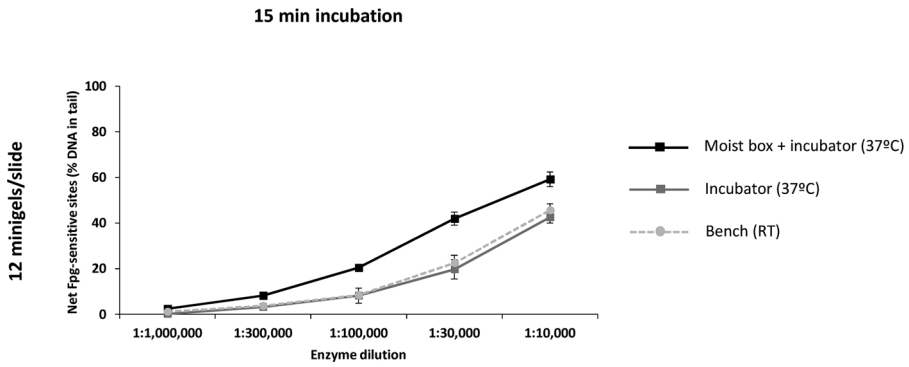

Fig. 3. Net Fpg-sensitive sites obtained after incubating 1.25 mM treated cells, after lysis, with different Fpg dilutions for 15 min and using the 12-Gel Comet Assay Unit™. The incubation was performed using 3 different strategies; the unit was included in a moist box and then placed it in the incubator at 37 °C, the unit was placed directly in the incubator, and the unit was left on the bench at room temperature. RT: room temperature.

incubation step. In the 2 gels/slide format, gels are incubated with the enzymes by adding a 50 µl drop on top of each gel (covered by a coverslip). Meanwhile, in the 12 minigels/slide format, the slide is put within the 12-Gel Comet Assay Unit™ and 30 µl of enzymes is added on each well. In both cases, the system allows to detect up to approximately 75% DNA in tail of net-Fpg sensitive sites. Results show that 10x lower enzyme concentrations are needed in the case of the 12 minigels/slide format compared with 2 gels/slide. This could be due to the higher ratio of enzyme volume/volume of gel; 0.7 for the 2 gels/slide format and 6 in the 12 minigels/slide one. Moreover, in the case of the 2 gels/slide format, the coverslip used to cover the drop of the enzyme spreads some volume of the enzyme outside the gels and some evaporation may occur.

Increasing the time of incubation from 15 to 30 min has a clear effect in both formats. However, increasing the time from 30 min to 1 h has a slight effect in the case of the 2 gels/slide format and a remarkable effect in the 12 minigels/slide format (at the lower enzyme concentration testes, the effect of time is remarkable in both formats). As mentioned before, in the case of the 2 gels/slide format, slides were placed on an ice-cold metal plate for the addition of the enzyme, transferred to a pre-heated moist box and the box was placed in the incubator at 37 °C. However, slides containing 12 minigels/slide are transferred to a cold 12-Gel Comet Assay Unit™, then the enzyme is added to each well of the unit (containing one gel) and the unit is transferred to a moist box. The unit is made of metal and it is quite bulky, so it may need more time to reach the proper temperature for the enzyme reaction. Actually, the unit is quite cold up to 10 min after being inside the moist box in the incubator (at 37 °C). This 'cooling effect' may be partly responsible of the necessity of longer incubation for a complete enzyme reaction. However, results obtained after 15 min of incubation showed that the enzyme is already acting when using both formats.

The 12-Gel Comet Assay Unit™ does not need to be placed inside a moist box, but can be placed directly within the incubator. In this case, the cooling effect is much higher; the unit is quite cold up to 45 min. In this regard, Fig. 3 shows that placing the unit directly in the incubator slows down the enzyme reaction since lower amounts of Fpg-sensitive sites are detected in comparison to placing the unit in a pre-heated moist box inside the incubator. Surprisingly, the enzyme is also active when the cold unit is left on the bench at room temperature. Actually, the same results are obtained after 15 min when leaving the unit on the bench or in the incubator. It is worth mentioning that if several cold units are included in the same moist box/incubator, the 'cooling effect' can be higher.

Other equipment can be used to perform the incubation of the enzymes, such as the 'slide moat' (e.g., from Boekel Scientific). Though this equipment is designed for the incubation of microscope slide, with a home-made frame it can be adapted to hold the 12-Gel Comet Assay Unit™. In this case, the 'cooling effect' is lower; the metal unit reached 37 °C after approximately 4 min of incubation (observation made by the authors).

On the other hand, the thickness of the gels may also be partly responsible for the longer incubation time needed in the case of the 12 minigels/slide format. Gels in the 2 gels/slide format are slimmer, since they are made by adding a coverslip on top a drop of cell suspension in agarose. In the case of the gels in the 12 minigels/slide format, a coverslip is not added so the gels have a dome shape. The enzyme, added to each well of the 12-Gel Comet Assay Unit™, may need some time to reach nucleoids in the thickest central part of the gel.

The 12 minigels/slide format was developed to increase the throughput of the assay, as 6 times as many samples can be analyzed in each experiment (i.e., one run of electrophoresis), fewer cells are needed, and, when using the enzyme-modified comet assay, less volume of enzyme/gel is also required [3]. Moreover, lower amounts of buffers are needed per sample in comparison with the 2 gels/slide format. To all these advantages (and some more included in the 1st paper about this format [3]) should be added the considerably lower concentration of enzyme that is required.

The 12 minigels/slide format, together with the 12-Gel Comet Assay Unit™, are the preferred format for the *in vitro* comet DNA repair assay [18]. This repair assay involves incubation of cell extract with nucleoids containing specific lesions, and so it is similar to the Fpg-modified assay described here. Therefore the results presented in this paper have to be taken into account, and care is needed in comparisons with results obtained with the 2 gels/slide format.

Titration experiments are crucial when the comet assay in combination with enzymes is performed. They should be done using a nucleoid substrate containing lesions appropriate for the enzyme being studied, as well as untreated substrate (from non-treated healthy cells) to detect the non-specific activity of the enzyme. It is crucial that the specific substrate contains a reasonable level of specific lesions and as low as possible levels of DNA strand breaks. The amount of lesions should be high enough without reaching the saturation level of the comet assay. In any case, a plateau should be reached to be sure that all the lesions have been detected. In our case, we have used substrate cells containing about 75% DNA in tail in terms of net-Fpg sensitive sites; since we are going to use the enzyme in genotoxicity testing we want to be sure that we can detect high levels of net-Fpg sensitive sites. So, even if the value of 75% is high, the assay in our hands is not saturated (It is worth mentioning that the levels of DNA damage found with buffer when titrating the enzyme were around 1.5% DNA in tail.) It may be safer to perform titration experiments with a slightly lower level of DNA damage, e.g. 65–70% tail DNA, which implies testing - in a preliminary experiment - different concentrations of the compound inducing the lesions.

It is not easy to find a good compound to produce a specific substrate. An assessment of possible positive controls for the Fpg- and hOGG1-modified comet assay was published by Møller et al. in 2018 [19]. Potassium bromate ($KBrO_3$) seems to be perfect for the titration of Fpg or the human 8-oxoguanine DNA N-glycosylase (hOGG1); at certain concentrations/times of incubation it induces a very high amount of 8-oxo-gua without inducing DNA strand breaks. This was observed before by Møller et al. in 2015 [20]. To find compounds to produce substrate to titrate other enzymes, as for example endonuclease III, can be very difficult. (Incubating cells with H_2O_2 and allowing them to repair SBs, leaving oxidised bases, may be the best approach available.)

Titration experiments must be done in house, using the same format and the same protocol and equipment that is going to be used in the forward experiments. It is worth mentioning that the duration of the lysis and the alkaline treatment also affect the detection of Fpg-sensitive sites [21,22]. Moreover, in the case of crude enzyme extracts, titration experiments should be repeated if a new batch of the enzyme is going to be used. In the case of the commercial ones and since the units of enzymes per mL are given in each batch, an adjustment of the concentration without repeating the titration experiment could be enough. However, to be sure about this, we also recommend to carry out titration experiments whenever a new batch of a commercial enzyme is to be used.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Chapter 5

Novel approach for the detection of alkylated bases using the enzyme-modified comet assay

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ABSTRACT

The enzyme-modified comet assay is widely used for the detection of oxidized DNA lesions. Here we describe for the first time the use of the human alkyladenine DNA glycosylase (hAAG) for the detection of alkylated bases. hAAG was titrated using untreated and methyl methanesulfonate (MMS)-treated TK-6 cells. The hAAG-modified comet assay was compared to the formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay, widely used to detect oxidized lesions but that also detects ring-opened purines derived from some alkylated lesions, using cells treated with potassium bromate (oxidizing agent) or MMS. Moreover, neutral and alkaline lysis conditions were used to determine the nature of detected lesions. When alkaline lysis was employed (condition normally used), the level of hAAG-sensitive sites was higher than the Fpg-sensitive sites in MMS-treated cells and hAAG, unlike Fpg, did not detect oxidized bases. After neutral lysis, Fpg did not detect MMS-induced lesions; however, results obtained with hAAG remained unchanged. As expected, Fpg detected oxidized purines and imidazole ring-opened purines, derived from N7-methylguanines under alkaline conditions. It seems that hAAG detected N7-methylguanines, the ring-opened purines derived at high pH, and 3-methlyladenines. Specificity of hAAG towards different DNA lesions was evaluated using a multiplex oligonucleotide-cleavage assay, confirming the ability of hAAG to detect ethenoadenines and hypoxanthine. The hAAG-modified comet assay is a new tool for the detection of alkylated bases.

1. Introduction

Alkylation is the process of covalent bonding of an alkyl group to a broad range of biological molecules, including nucleic acids. Alkylation can occur by a simple addition reaction or by substitution of another functional group. Alkylating agents are typically highly reactive and unavoidable, as they are broadly ubiquitous, being present both in the environment and endogenously within living cells (Fu et al., 2012).

There are different sources of alkylating compounds in the environment, such as pollutants that may be present in food, water or air (e.g. some tropical plants and fungi produce chloromethane, and some methylating agents are generated in tobacco smoke or fuel combustion products) (Ballschmiter, 2003; Hamilton et al., 2003; Ma et al., 2019). Moreover, alkylating compounds comprise a major class of cytostatic drugs in cancer therapy (Hurley, 2002; Drabløs et al., 2004). Additionally, alkylating compounds can also be found endogenously, arising as byproducts of oxidative stress or during metabolism; for instance, S-adenosylmethionine, a physiological methyl radical donor in enzymatic reactions *in vivo* (Rydberg and Lindahl, 1982).

On DNA, alkylating agents may form adducts at all oxygen and nitrogen atoms of the DNA bases, generating a variety of lesions with different complexity and implications in living cells, including cytotoxicity and mutagenicity (Drabløs et al., 2004; Shrivastav et al., 2009). Depending on the position of these alterations in DNA, the adducts induced by alkylating agents may pose a threat to genome integrity, as an unrepaired or erroneously repaired DNA lesion may lead to a mutation (thereby promoting carcinogenic processes) and/or may block essential biological processes (DNA replication or transcription). Additionally, it is worth mentioning that some lesions can be processed into byproducts that can also be clastogenic or cytotoxic (Fu et al., 2012).

N-methylation adducts are the most common alkylated bases, N7-

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methylguanine being the predominant adduct, comprising between 60–80 % of the total alkylation lesions in DNA (Shrivastav et al., 2009). In general, these N-alkylations (e.g., N7- and N1-methylguanine or N3-methyladenine) are mainly cytotoxic (blocking DNA polymerases and DNA synthesis), being less mutagenic compared to O-alkylations (Kondo et al., 2010), which are generated to a much lesser extent and are of great biological significance. O6-methylguanine is a primary mutagenic lesion under most conditions of alkylation damage to DNA, as it induces G-A transitions during replication; and O4-methylthymine is a mispairing lesion that also presents mutagenic potential (Shrivastav et al., 2009; Fu et al., 2012).

Nowadays there are several methods available for measuring different lesions in DNA, such as chromatographic techniques, the comet assay, polymerase chain reaction assays, mass spectrometry, electrochemistry, radioactive labeling, immunochemical methods or different sequencing methods (reviewed in Himmelstein et al., 2009). However, all have some limitations and only a few are applicable to the detection of alkylation damage in DNA.

The alkaline comet assay (single cell gel electrophoresis) is a widely used method for measuring DNA damage at the single cell level (Azqueta and Collins, 2013). Particularly, it detects strand breaks (SB) and alkali-labile sites (ALS) such as apurinic or apyrimidinic (AP) sites. It is relatively simple, economical and very versatile, as it can be applied to almost any eukaryotic cell type or to disaggregated tissues. Briefly, cells are embedded in agarose on a microscope slide and then lysed to form nucleoids, which contain supercoiled DNA. Lysed cells are further subjected to alkaline pH incubation to unwind the DNA prior to electrophoresis. Finally, naked DNA of individual cells is evaluated under the microscope: if DNA contains any SB, supercoiling will be disrupted so part of the DNA will migrate during the electrophoresis giving a comet-like image; whereas if DNA remains intact, supercoiling will be preserved and migration will not occur.

During the last three decades, the assay has been modified to detect other lesions, mainly oxidized bases, by the use of DNA-repair enzymes (DNA glycosylases), such as formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III), which are the most frequently used. These enzymes are able to detect and remove the base, leaving an AP-site, which is then converted to a SB by an associated AP lyase activity of the enzyme (Azqueta and Collins, 2013). In practice, enzyme digestion is applied to the nucleoids that are formed after lysing the agarose-embedded cells. A digestion with enzyme buffer alone gives a measure of SBs and ALS; the difference between comet scores for the two gels, +buffer and +enzyme, indicates the frequency of 'net enzyme-sensitive sites' (Azqueta et al., 2013a). Traditionally, this enzyme-modified comet assay has been applied mainly for the detection of oxidized bases (Collins, 2014).

However, specific enzymes for the detection of alkylated bases have also been applied in combination with the comet assay. The enzyme 3-methyladenine DNA glycosylase II (AlkA) is a bacterial repair enzyme with 3-methyladenine as its main substrate. It was first reported in combination with the comet assay by Collins et al. (2001). Similarly, the enzyme 3-methyladenine DNA glycosylase (AlkD) is also a bacterial repair enzyme that was applied in combination with the comet assay for the first time by Hašplová et al. (2012). It is specific for 3-methyladenine and 7-methylguanine. However, according to our knowledge, these enzymes are not commercially available and so the majority of researchers do not have access to them or the facilities and knowledge to produce them. Actually, these enzymes have not been extensively used and there are no recent publications showing their use in combination with the assay; there are a total of 16 publications using AlkA, most of them from the same group, and only 2 publications using AlkD).

It is worth mentioning that Fpg, apart from detecting oxidized bases, also detects ring-opened purines derived from some alkylation lesions at alkaline conditions (i.e., during lysis at pH 10 in the comet assay) (Speit et al., 2004; Smith et al., 2006; Azqueta et al., 2013a; Hansen et al., 2018).

Here we describe, for the first time, the use of the human enzyme alkyladenine DNA glycosylase (hAAG), a commercially available enzyme, in combination with the comet assay to detect alkylated bases on DNA of human lymphoblastoid cells (TK-6 cell line). This enzyme, also known as methylpurine DNA glycosylase (MPG) and alkyl-N-purine DNA glycosylase (ANPG), is the enzyme initiating the base excision repair (BER) pathway for the repair of alkylation adducts. In particular, it detects 3-methyladenine and 7-methylguanine (O'Connor, 1993). hAAG is a monofunctional glycosylase that catalyzes the hydrolysis of the N-glycosidic bond, releasing the N-alkyl-adduct from DNA and leaving an abasic site that can be detected using the comet assay (Lau et al., 1998). Moreover, the Fpg-modified and the hAAG-modified comet assay are compared under two different lysis conditions (i.e., pH 7 and pH 10) and the activity of hAAG in detecting other lesions was determined by using the Glyco-SPOT assay (multiplex oligonucleotide-cleavage assay).

2. Material and methods

2.1. Chemicals and reagents

Low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, Bovine Serum Albumin (BSA), NaOH, KCl, potassium bromate ($KBrO_3$) methyl methanesulfonate (MMS) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. DPBS 1x for mixing cell suspensions with agarose was purchased from Gibco. DPBS without Ca^{+2} and Mg^{+2} 10x from Lonza was used to prepare PBS 1x washing solutions for comet assay slides. Dimethyl sulfoxide was purchased from PanReac AppliChem. All cell culture reagents were purchased from Gibco.

hAAG and Endo III were purchased from New England Biolabs (catalog number M0313S and M0268S respectively). Fpg from an overproducing *E. coli* strain was kindly provided by NorGenoTech AS (Oslo, Norway).

2.2. Cell culture

The human-derived lymphoblastoid TK-6 cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI (Roswell Park Memorial Institute, ref. A10491-01, Gibco) medium containing D-glucose, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate and supplemented with 10 % heat-inactivated fetal calf serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Gibco). Cells were maintained as a suspension culture in continuous agitation at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were maintained in culture for no longer than 2 months.

2.3. Treatment and freezing of cells

TK-6 cells were seeded at 1×10^6 cells/mL in culture medium containing no serum and treated for 3 h with different non-cytotoxic concentrations of either MMS or $KBrO_3$ or their vehicles (DMSO and water respectively). Using these conditions, preliminary studies were performed to assess cytotoxicity employing the proliferation assay according to Azqueta et al. (2013a) and concentrations with a relative suspension growth (RSG) over 80 % were selected for further experiments. Concentrations for specific purposes are detailed in their respective sections. Cells were kept in continuous agitation at 37 °C in a humidified atmosphere with 5 % CO_2.

From this point, cells were kept cold to prevent DNA repair. After treatment, cells were centrifuged and washed twice with phosphate-buffered saline (PBS). Then cells were resuspended in culture medium containing 5 % DMSO and aliquoted in cryotubes at 1×10^6 cells/mL in 0.5 mL. Finally, cells were frozen by using the freezing container Mr. Frosty (Thermo Scientific, Nalgene). The container including the cryotubes was placed in a freezer at −80 °C at least overnight. After

that, cryotubes were transferred to boxes and kept at −80 °C until analysis.

2.4. hAAG-modified comet assay

2.4.1. Titration

The comet assay was performed using the medium-throughput format of 12-Gel Comet Assay Unit™ (Shaposhnikov et al., 2010) as previously described (Muruzabal et al., 2018). To titrate the enzyme, frozen cells (untreated and 1.25 μM MMS-treated cells; previous section) were employed as substrate. MMS was employed as it is a known alkylating agent. Frozen cells were quickly thawed by immersing the cryovial in a water bath at 37 °C and washed in 10 mL of cold PBS by centrifugation. Then cells were suspended in PBS at 2.5×10^5 cells/mL. For the preparation of the agarose minigels, 30 μL of cell suspension were mixed with 140 μL of 1 % low melting point agarose in PBS at 37 °C. After that, 12 droplets of 5 μL each of the corresponding cell suspension were placed on agarose-precoated slides. Slides were placed on the bottom metal holder of the 12-Gel Comet Assay Unit™ (NorGenoTech, Oslo, Norway), previously cooled in the fridge, which contains a template to set the minigels in certain positions (two rows of six).

Once gels were prepared, slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 0.1 M Tris base, pH 10 and 1 % Triton X-100) at 4 °C for 1 h. Prior to enzyme/buffer treatment, slides were washed three times, 5 min each at 4 °C with the reaction buffer of the enzyme (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8).

During the washes, hAAG was prepared by making serial dilutions: in preliminary studies a broad range of enzyme dilutions (i.e., from 1:100 to 1:1,000,000 using a dilution factor of 10) was employed and the range was then reduced to 1:300 − 1:100,000; with 1:3 and 1:3.33 dilution factors. The enzyme was diluted from the original stock (10,000 U/mL) using the reaction buffer. After washing with the enzyme reaction buffer, slides were transferred to a cold 12-Gel Comet Assay Unit™ (12-Gel Comet Assay Unit™, NorGenoTech, Oslo, Norway). These units allow differential treatments, as gels are isolated in wells on each slide. Units were placed on a cold metal plate to keep them cold during the enzyme or buffer addition to avoid enzymatic reactions until incubation. Thirty microliters of hAAG enzyme or reaction buffer were pipetted to each well and a clean slide was placed on top of the unit to cover all wells and prevent contamination and evaporation. The design of each 12-minigels slide was the same in all cases: 2 minigels were incubated with enzyme buffer alone and 5 concentrations of hAAG were tested on 2 minigels each. The 12-Gel Comet Assay Units™ were then transferred to a pre-heated moist box and placed in the incubator at 37 °C. To detect the optimal incubation time, initially different times of incubation were employed (15 min, 30 min, 45 min, 1 h and 1 h 15 min).

After incubation, the enzyme reaction was stopped by placing the units on a cold plate. Subsequently, slides were transferred to the electrophoresis tank for unwinding in electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH > 13) during 40 min at 4 °C. Afterwards, electrophoresis was carried out at 1.2 V/cm for 20 min (4 °C).

Following electrophoresis, slides were neutralized by washing them in PBS for 10 min and then in distilled water for 10 min. To dehydrate the gels for avoiding an edge effect (i.e. comets going in different angles) (Azqueta et al., 2013b), slides were immersed in 70 % ethanol for 15 min and then in absolute ethanol for 15 min. Finally, slides were air dried at room temperature overnight.

For scoring the comets, each gel was stained with a 5 μL drop of 1 μg/mL of 4,6-diamidino- 2-phenylindole (DAPI) and a coverslip (24 × 60 mm) was placed on top to cover all the minigels of a slide. After 30 min of incubation with DAPI at room temperature, slides were analyzed using the semi-automated image analysis system Comet Assay IV (Perceptive Instruments) and 50 nuclei per gel, 100 per condition, were scored. The percentage of DNA in tail (or tail intensity) was used as descriptor for each comet, and the median percentage tail DNA of 100 comets was taken as the measure of DNA damage for each condition. Net enzyme-sensitive sites were calculated by subtracting the percentage tail DNA obtained with the buffer incubation alone from that obtained after hAAG incubation at different concentrations. To determine the optimal concentration and incubation time for the enzyme, relative activity of hAAG was calculated by subtracting the level of DNA damage of non-treated cells from the level of the MMS-treated ones.

Titration experiments using 12-Gel Comet Assay Unit™ were performed twice (independent experiments).

2.4.2. Measuring different levels of alkylated bases

The hAAG-modified comet assay using different concentrations of hAAG (i.e., 33.33, 10, 3.33. 0.33 and 0.1 U/mL) and 1 h of incubation was applied to cells containing different levels of alkylated lesions. In particular, cells treated with a range of non-cytotoxic concentrations of MMS (0, 1.25, 2.5, 3, 4 and 10 μM) were employed to test whether higher levels of DNA damage could be detected. At least three independent experiments (four in some cases) were performed using the 12-Gel Comet Assay Unit™ with each of the MMS concentration.

A concentration-response curve using the selected conditions for hAAG incubation, 10 U/mL of enzyme and 1 h of incubation time at 37 °C was constructed.

2.5. hAAG- vs Fpg-modified comet assay

2.5.1. Concentration-response experiments with MMS and $KBrO_3$

Performance of hAAG and a crude extract of Fpg (from an overproducing *E. coli* strain) was compared towards different substrates using the 12-Gel Comet Assay Unit™. The comet assay was performed as previously described using the optimized conditions for the incubation with hAAG (10 U/mL and 1 h at 37 °C) employing cells treated with either MMS (0, 5, 10 and 20 μM) or $KBrO_3$ (0, 0.313, 0.625 and 1.25 mM) for 3 h. Fpg was previously titrated (Muruzabal et al., 2018). The same reaction buffer was used for both enzymes. Two independent experiments were performed.

2.5.2. Activity in MMS- and $KBrO_3$- treated cells using neutral and alkaline lysis

Performance of hAAG and a crude extract of Fpg (from an overproducing *E. coli* strain) was studied using neutral and alkaline lysis conditions in the comet assay. These experiments were performed in a different laboratory using a high throughput (HTP) comet assay format with Gelbond® films (Cambrex, Rockland, ME) as a matrix. For this reason, the enzyme was also titrated with this format. This HTP version was performed according to Hansen et al., 2010 and Gutzkow et al., 2013 with some modifications; unless otherwise indicated recipes of the solutions and buffers were the same as explained in Section 2.4.1. Briefly, cells were mixed with 1 % low melting point agarose dissolved in PBS at 37 °C and moulded as 48 gels (4 μL each) per GelBond® film (being 0.82 % the final agarose concentration). The moulding process was performed over metallic plates that were previously cooled in the fridge at 4 °C. Films were immersed immediately in lysis solution (pH 10) for 1 h. Prior to enzyme incubation, films were washed three times (5 min each) with enzyme buffer at 4 °C. Commercial hAAG enzyme was titrated using five dilutions (1:1,000 − 1:100,000 using 1:3 and 1:3.33 as dilution factors) from the original stock using only 1 h as incubation time. Incubation was performed by immersing the films in trays with enzymes diluted on enzyme buffer for 1 h at 37 °C. After enzyme treatment, electrophoresis was performed at 0.85 V/cm at 4 °C and with a solution recirculation system. The remaining procedure was the same as aforementioned except for film staining for scoring. Staining was performed with 1:12,500 SYBR Gold nucleic-acid gel stain (Invitrogen, Oslo, Norway) in TE buffer (10 mM Trizma HCl and 1 mM Na_2EDTA, pH 8) for 20 min with shaking in the dark.

Once conditions were optimized, untreated cells and cells treated with either MMS (2.5, 5 or 10 μM) or $KBrO_3$ (0.5, 1 or 2 mM) for 3 h

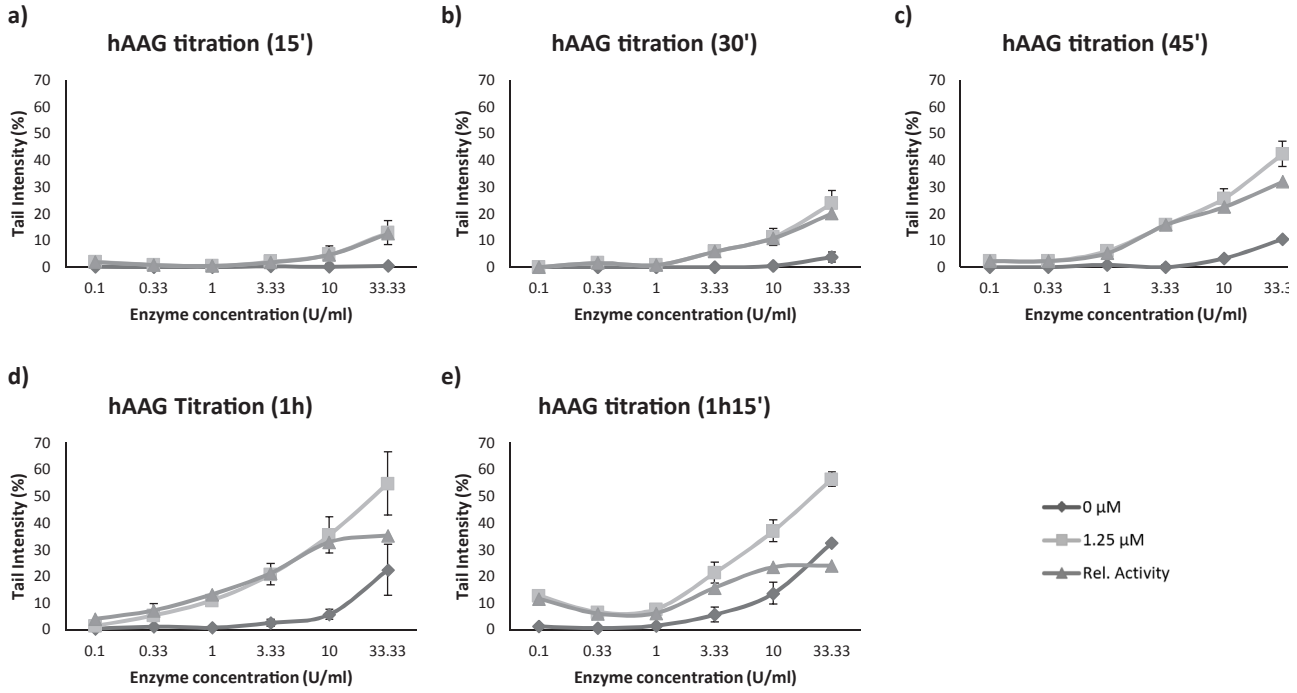

Fig. 1. *hAAG titration using the 12-Gel Comet Assay Unit™.* Titration curves for the different incubation times tested: a) 15 min, b) 30 min, c) 45 min, d) 1 h and e) 1 h and 15 min. Results of 0 and 1.25 µM MMS are expressed as net enzyme-sensitive sites (in terms of tail intensity); relative activity of hAAG was calculated by subtracting the level of DNA damage of non-treated cells from the level of treated ones. Data from two independent experiments are presented (mean and individual experimental values).

were employed as substrate. The HTP version of the comet assay employing GelBond® films was used following the protocol describe in Section 2.4.3 for the titration of hAAG. Additionally, lysis buffers with different pH were used. To do so, a duplicate of each film was prepared, and then half of the films were immersed in basic lysis solution (pH 10), and the remaining films were immersed in neutral lysis solution (pH 7) for 1 h; the remaining procedure was the same as previously described. hAAG enzyme was employed at 1 U/mL and Fpg at 0.5 µg/mL (as it was a home-made stock, the amount of units was not determined) and the incubation time with both enzymes was 1 h at 37 °C. Two independent experiments were performed.

2.6. Glyco-SPOT assay

The Glyco-SPOT assay is a multiplexed ODN (oligonucleotide) cleavage assay on support and it was used to simultaneously control the activity of hAAG toward several potential substrate lesions. 24-well glass slides (Streptavidin-coated, Xantec bioanalytics, Germany) functionalized with a panel of ODNs bearing different lesions (8oxoG paired with C -8oxoG-C-, A paired with 8oxoG -A-8oxoG-, ethenoadenine paired with T -EthA-T-, hypoxanthine paired with T -Hx-T-, tetrahydrofuran -abasic site stable analog- paired with A -THF-A-, thymine glycol paired with A -Tg-A-, and Uracil paired either with G or with A -U-G and U-A, respectively-) and labelled with a Cy3 at their end, were used as described in Candéias et al., 2010; Pons et al., 2010. Each ODN was immobilized in duplicate in each well together with a Control-ODN that contained no modification. Five different concentrations of hAAG were tested (final concentration: 0.007 U, 0.02 U, 0.07 U, 0.2 U, 0.6 U) in two different wells in the same enzyme-reaction buffer as employed for the enzyme-modified comet assay (for details see Section 2.4.1). As hAAG is a monofunctional enzyme, it requires an additional AP-endonuclease activity to cleave the abasic site resulting from the cleavage of the N-glycosidic bond (Lau et al., 1998). Consequently, the removal of the damaged base by hAAG was revealed through the addition of Endo III (0.5 U/well) that catalyzed the cleavage of the abasic site. (In the comet assay, cleavage is achieved by the alkaline conditions during unwinding and electrophoresis.) The activity of Endo III was initially titrated to select the most adapted concentration. Indeed, it was active only against thymine glycols. The excision reaction was run for 60 min at 37 °C under agitation (700 rpm). Then the slides were washed 2 × 5 min in 1XPBS containing 0.2 M NaCl - 0.05 % Tween 20 and dried by centrifugation.

For each spot, fluorescence was quantified at 532 nm wavelength using the Innoscan 710AL scanner from Innopsys (Toulouse, France) and the associated MAPIX software. Data were normalized as described using NormalizeIt software (Millau et al., 2008). To calculate the final cleavage rate of each ODN-containing lesion, the fluorescence of the control well, incubated with the excision buffer only, was taken as reference (100 % fluorescence). The data were also corrected by the control-ODN cleavage rate that remained below 10 %. Finally, the lesion-ODN cleavage percentage was 100 x (1 - percentage of fluorescence of lesion-ODN/percentage of fluorescence of control-ODN).

2.7. Statistics

The median percentage of DNA in tail for 50 comets was calculated for each of the duplicate minigels in each experiment; the mean of the two medians was then calculated. We show the mean percentage of DNA in tail for the duplicate experiments together with the individual experimental values in the titration experiments and in the experiments comparing the performance of hAAG- and Fpg-modified comet assay. The mean and the SD of the 3 or 4 independent experiments performed when testing the capability of hAAG to detect different levels of alkylated lesions are shown.

3. Results

3.1. hAAG-modified comet assay

3.1.1. Titration of hAAG

For hAAG titration, untreated and 1.25 μM MMS-treated cells were used as substrate and the assay was performed with the 12-Gel Comet Assay Unit™. To select an appropriate range of hAAG dilutions to test, preliminary studies were performed with a broad range of enzyme dilutions (data not shown) to select the final range presented here (0.1–33.33 U/mL).

Fig. 1 shows the titration curves of hAAG after 15, 30 and 45 min, 1 h and 1 h 15 min of incubation with nucleoids from MMS-treated and untreated cells. In this figure, net hAAG-sensitive sites obtained in treated and untreated together with the relative activity of the enzyme are presented. MMS-treated cells showed a clear enzyme concentration-response at all incubation times tested, especially after 45 min, and reached the maximum level of enzyme-sensitive sites detected at 1 h of incubation; after 1 h 15 min of incubation the detected DNA damage on treated cells did not increase further. Regarding non-treated cells, hAAG-sensitive sites were found at the highest concentration tested (33.33 U/mL) in all incubation times from 45 min onwards (Fig. 1*c*–*e*). A clear increase of breaks was also found at lower concentrations of enzyme after 1 h 15 min of incubation (Fig. 1*e*), suggesting a non-specific nuclease activity.

When studying the relative activity of the enzyme, a plateau was reached at almost 40 % of DNA in tail after 1 h of incubation time with the two highest concentrations tested (i.e. 10 and 33.33 U/mL) (Fig. 1*d*). Indeed, it was the only incubation time along with 1 h 15 min in which a plateau was reached. However, after 1 h 15 min of incubation, the plateau of relative activity was reached at lower levels of DNA damage (20 % of DNA in tail) (Fig. 1*e*).

Thus, the following conditions were selected as optimal for the hAAG-modified comet assay: 10 U/mL of enzyme and 1 h as incubation time. Thus, according to these results the range of hAAG-sensitive sites that can be detected goes from 0 to at least 30 % of DNA in tail.

3.1.2. Measuring different levels of alkylated bases

Following the titration experiments, to evaluate whether hAAG could detect high levels of alkylated lesions, the hAAG-modified comet assay was performed using a range of concentrations of the enzyme with cells treated with different non-cytotoxic concentrations of MMS (0, 1.25, 2.5, 4 and 10 μM, RSG > 80 %). These experiments were performed using the 12-Gel Comet Assay Unit™. Fig. 2 shows the net enzyme-sensitive sites obtained for each condition (Fig. 2*a*), the relative activity for each enzyme concentration (Fig. 2*b*) and the resulting concentration-response curve using the selected conditions for hAAG (i.e., 10 U/mL of enzyme and 1 h of incubation time at 37 °C) (Fig. 2*c*).

As expected, the highest hAAG concentration tested (i.e. 33.33 U/mL) produced breaks in untreated cells, whereas no significant levels of enzyme-sensitive sites were detected at the remaining concentrations (Fig. 2*a*). However, a slight increase (5 % of DNA in tail) was observed using hAAG at 10 U/mL (Fig. 2*a*).

Regarding MMS-treated cells, a clear concentration-response pattern was observed in all MMS concentrations tested. The assay was saturated at the highest MMS concentration (10 μM) from 3.33 U/mL of hAAG (Fig. 2*a*).

The relative activity of the enzyme was also calculated for each hAAG concentration at all conditions tested (Fig. 2*b*). As shown in the figure, a plateau is reached at 10 U/mL of hAAG in the case of MMS concentrations of 1.25 and 1.5 μM. With higher MMS-concentrations, the relative activity with 33.33 U/mL of enzyme decreased considerably; this effect is accounted for by the high levels of unspecific DNA damage in non-treated cells combined with the saturation of the assay in the treated ones.

A concentration-response curve showing the activity of hAAG under selected conditions (i.e., 10 U/mL and 1 h of incubation) against substrates treated with different MMS concentrations was also constructed (Fig. 2*c*). The figure shows that with 10 U/mL of hAAG it was possible to detect MMS-induced alkylating damage from low concentrations up to the upper detection limit of the assay (i.e., about 80 or 90 % of DNA in tail). Despite the marginal unspecific damage induced in non-treated cells, it was the only concentration able to detect such levels.

In these experiments, the enzyme reaction buffer alone did not induce DNA damage in any of the conditions tested (data not shown), which indicates the absence of SB.

3.2. hAAG- vs Fpg-modified comet assay

3.2.1. Concentration-response experiments with MMS and $KBrO_3$

The activities of hAAG and Fpg were compared using the 12-Gel Comet Assay Unit™. For this purpose, cells were treated at three non-toxic concentrations of either MMS, an alkylating agent, or $KBrO_3$, an oxidizing compound. The net enzyme-sensitive sites obtained with both enzymes are shown in Fig. 3.

When cells treated with different concentrations of MMS were analyzed (Fig. 3*a*), both enzymes showed a concentration-dependent increase in tail intensity representing the detection of alkylated lesions. However, hAAG detected considerably higher levels of DNA damage, as it reached the upper detection limit of the assay (i.e., about 80 or 90 % of DNA in tail), even at the lowest MMS concentration tested (5 μM). Regarding the oxidizing compound (Fig. 3*b*), a concentration-dependent increase in DNA damage was observed with Fpg, whereas no damage was detected with hAAG. As it can be seen in Fig. 3, in these experiments the enzyme reaction buffer alone did not induce DNA damage in any of the conditions tested, which indicates the absence of SB.

3.2.2. Activity in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis

A HTP format of the comet assay using GelBond® films was used to carry out these experiments. To apply the hAAG-modified comet assay in this format, titration experiments were carried out in untreated and MMS-treated cells using 1 h of incubation and 5 different hAAG concentrations (i.e., 0.1, 0.33, 1, 3.33 and 10 U/mL). Results showed that 1 U/mL obtained the highest relative activity without inducing unspecific damage in non-treated cells (data not shown). This hAAG concentration and 1 h of incubation were used in the following experiments.

The Fpg- and hAAG-modified comet assay was applied in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis solution to elucidate the type of lesions detected. Lysis at alkaline pH converts some alkylated bases into ring-opened purines (Speit et al., 2004). The net enzyme-sensitive sites obtained for both enzymes in each experimental condition are shown in Fig. 4. As can be observed, the pH during the lysis step did not affect the level of MMS-induced DNA lesions detected with hAAG at any of the concentrations tested (Fig. 4*a* and *c*). The net-hAAG sensitive sites increased depending on the MMS concentration. However, the concentration-dependent increase observed in the levels of Fpg-sensitive sites when using an alkaline lysis (*Fig. 4 a*) was not observed when using neutral lysis (Fig. 4*c*). In this case, the Fpg-sensitive sites detected with the different MMS concentrations tested were the same as in untreated cells.

To confirm that this reduction in the pH of the lysis solution did not affect oxidized lesions, cells were also treated with different concentrations of $KBrO_3$ (Fig. 4*b* and *d*). In this case, pH did not affect the levels of either hAAG- or Fpg-sensitive sites in $KBrO_3$-treated cells. As expected, hAAG did not detect the induced lesions whereas a concentration-dependent increase of net fpg-sensitive was observed.

Untreated cells in both conditions (MMS and $KBrO_3$ experiments) showed a higher level of Fpg-sensitive sites (around 15 % of DNA in tail) than hAAG-sensitive sites (0–3 % of DNA in tail). No SB were detected with any of the compounds at any of the tested concentrations

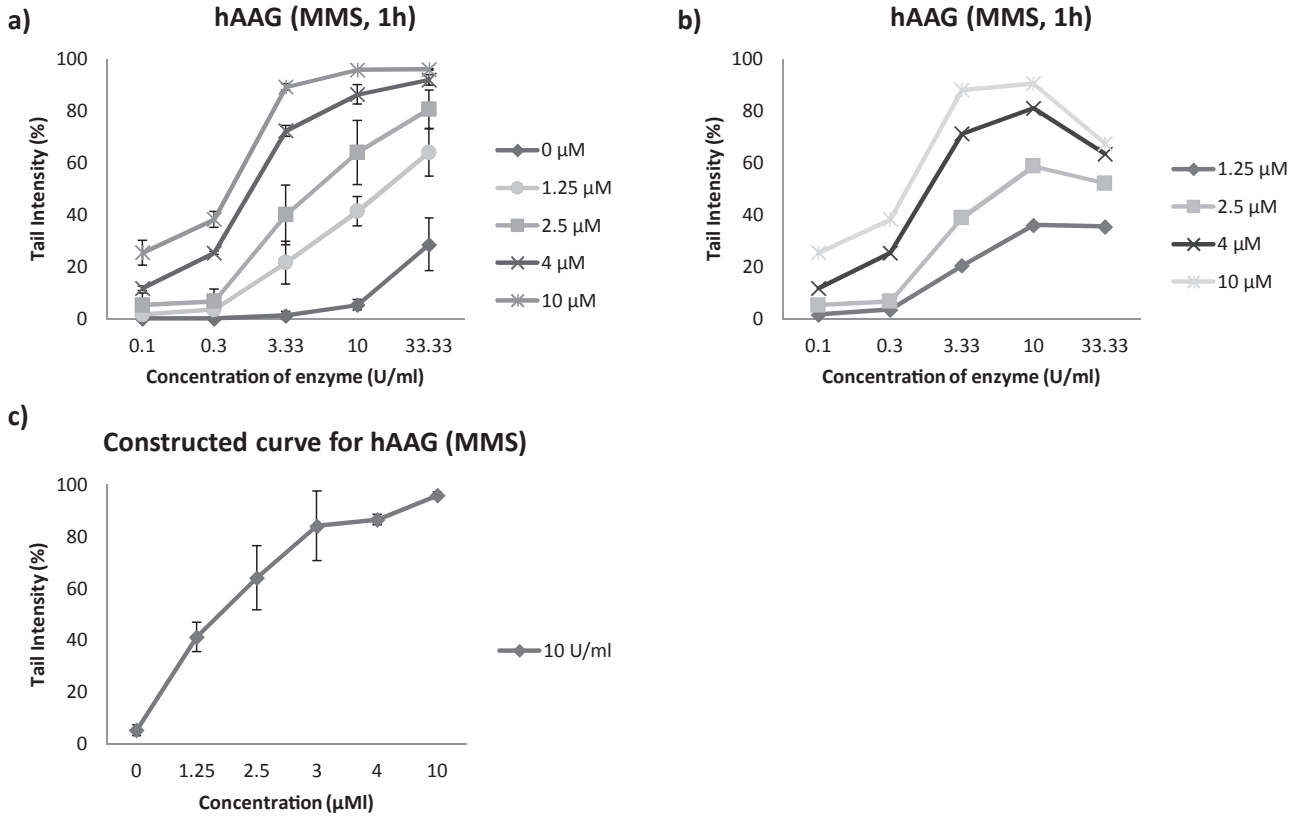

Fig. 2. *Measuring different levels of alkylated damage*. a) net enzyme-sensitive sites (in terms of tail intensity) obtained for each MMS concentration tested; b) relative activity of each enzyme concentration for each MMS concentration tested and c) MMS concentration-dependent curve of the net hAAG-sensitive sites using the selected conditions (10 U/mL and 1 h of incubation). Data from three independent experiments (4 in some cases) are presented (Mean ± SD).

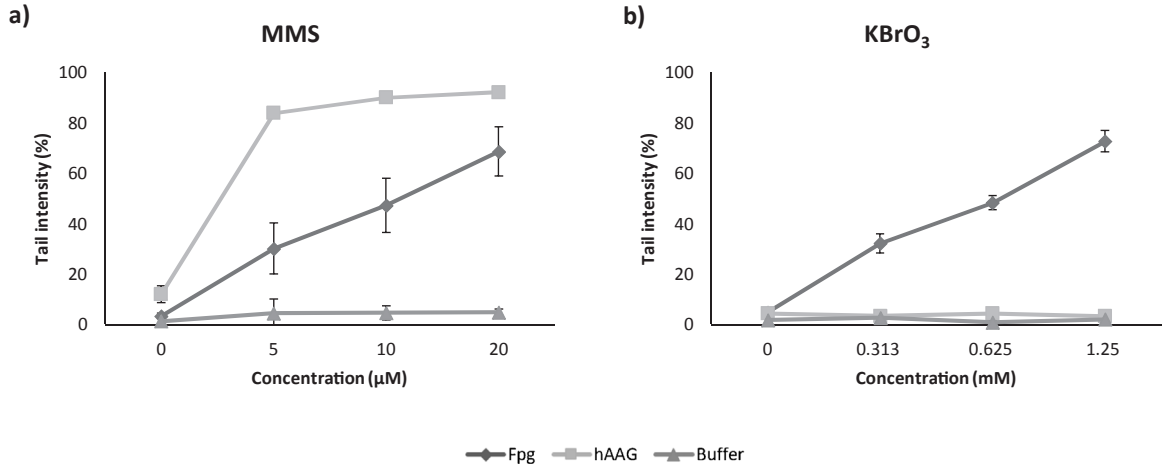

Fig. 3. *Concentration-response curves after performing the hAAG- and Fpg-modified comet assay in $KBrO_3$- and MMS- treated cells*. Results are expressed as net enzyme-sensitive sites (in terms of tail intensity) and the level of SB induced by the buffer alone ("Buffer"); a) MMS curve and b) $KBrO_3$ curve. Data from two independent experiments are presented (Mean and individual experimental values).

(Fig. 4). Indeed, SB levels were not affected by the variations in the pH of the lysis solution with any of the compounds at the concentrations tested.

3.3. Glyco-SPOT assay

Incubation of the different lesions with increasing concentrations of hAAG + Endonuclease III (Endo III) 0.5 U resulted essentially in the cleavage of ethenoadenine and thymine glycol. Hypoxanthine, which is also a known substrate for hAAG, was also cleaved but only at the highest hAAG concentration (Fig. 5).

4. Discussion

Here, we describe for the first time the use of hAAG in combination with the comet assay for the detection of alkylated bases on DNA. Particularly, hAAG detects 3-methyladenine and 7-methylguanine (O'Connor, 1993; and according to the enzyme specification). Some studies have also reported that hAAG also detects 1-methylguanine (Lee et al., 2009). Recently, we showed the importance of titrating the

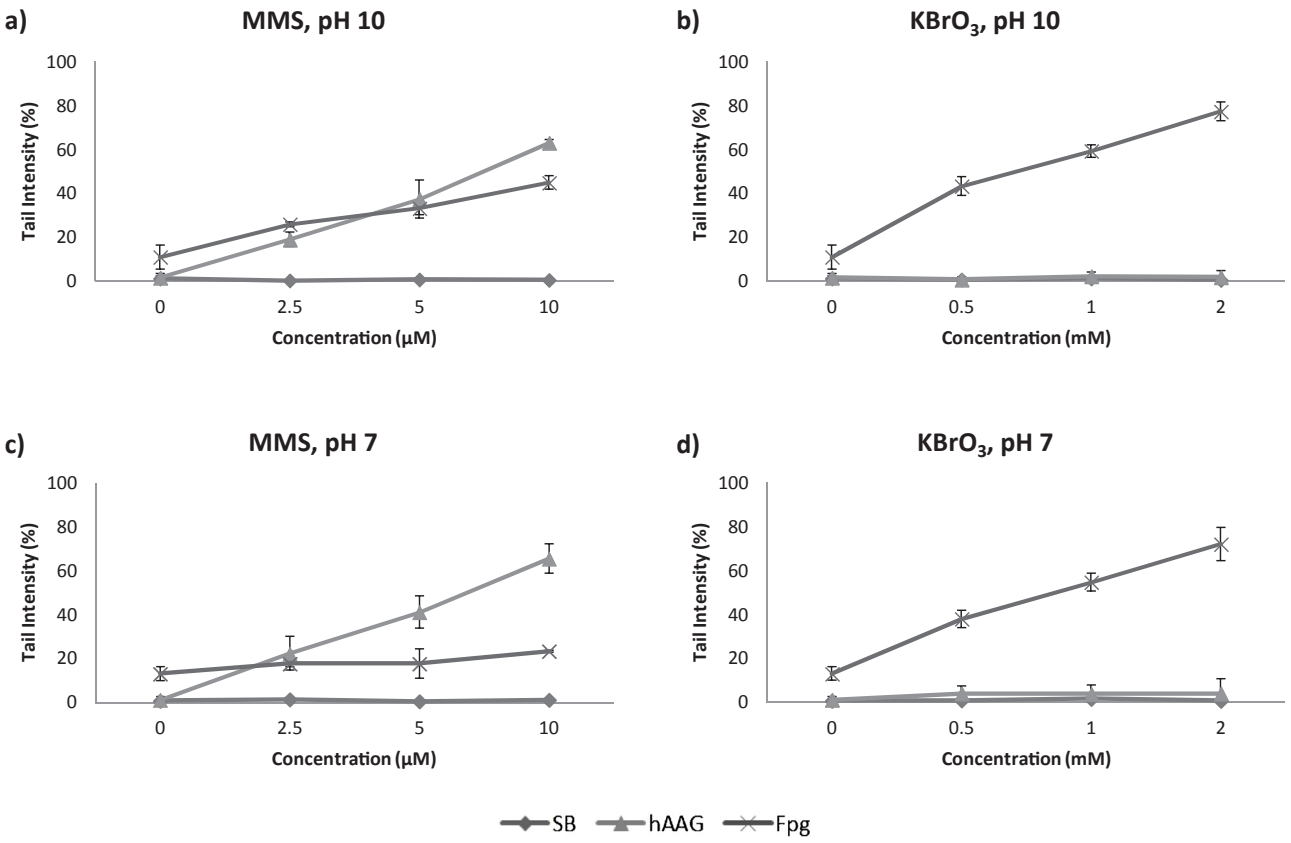

Fig. 4. *Activity of hAAG and Fpg in MMS- and $KBrO_3$-treated cells using neutral and alkaline lysis.* Results are expressed as net enzyme-sensitive sites (in terms of tail intensity) obtained for both enzymes and the SB obtained for each experimental condition. Alkaline lysis results with each compound are shown in a) and b); neutral lysis results are shown in c) and d). Data from two independent experiments are presented (Mean and individual experimental values).

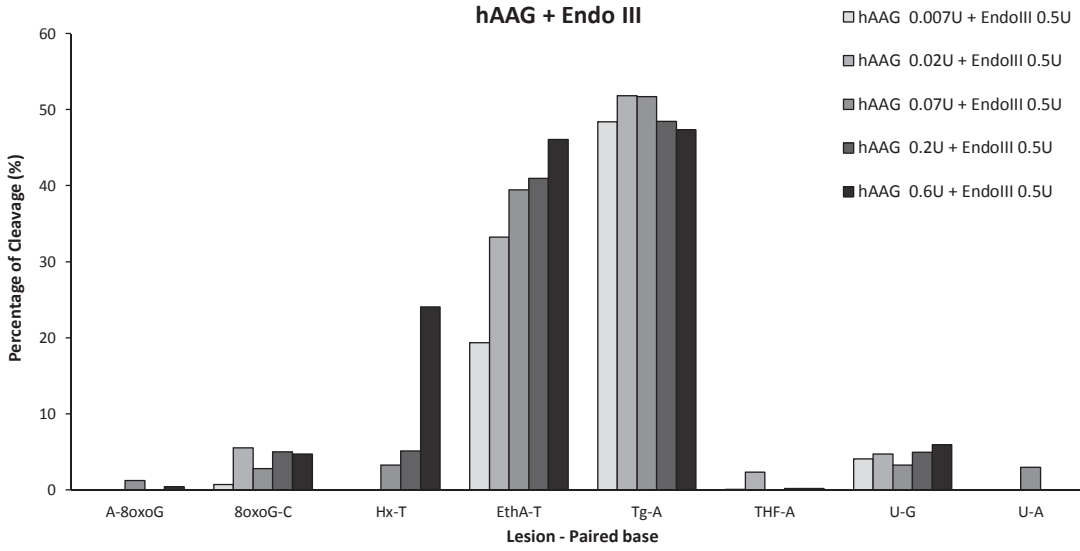

Fig. 5. *Percentage of cleavage induced by the hAAG (+Endo III) in the different DNA lesions included in the Glyco-SPOT assay*. A-8oxoG: A paired with 8oxoG; 8oxoG-C: 8oxoG paired with C; Hx-T: hypoxanthine paired with T; EthA-T: ethenoadenine paired with T; Tg-A: thymine glycol paired with A; THF-A: tetrahydrofuran -abasic site stable analog- paired with A; U-G: uracil paired with G; and U-A: uracil paired with A.

enzymes employed with the comet assay, taking into account the variations in comet assay format used (e.g., 2 gels/slide, 12 minigels/slide, etc) and incubation procedure (Muruzabal et al., 2018). Therefore titration experiments were conducted to determine the optimal hAAG concentration and time of incubation for detecting the maximum amount of enzyme-sensitive lesions without inducing non-specific breaks.

In this work, we have titrated commercial hAAG enzyme using the 12 minigels/slide format, and the 12-Gel Comet Assay Unit™ for incubation, in untreated and MMS-treated TK-6 cells. MMS is a known monoalkylating agent that induces mainly 7-methylguanine and 3-methlyladenine (Beranek, 1990). hAAG was successfully applied in

combination with the comet assay, and the optimal activity was obtained using 10 U/mL hAAG and 1 h of incubation; higher enzyme concentrations and longer time of incubation induced unspecific damage (Fig. 1). These conditions allow the detection of a wide range of alkylating damage, from the very low control levels up to the saturation level of the comet assay (Fig. 2*c*).

It is known that some alkylated lesions (i.e, N7-methylguanines) are prone to be converted into ring-opened purines at alkaline pH (Speit et al., 2004; Smith et al., 2006). Indeed, Gates (2009) reviewed different publications in which alkaline conditions were employed to accelerate the formation of alkyl-Fapy derivatives. This is observed when using the (alkaline) Fpg-modified comet assay. Fpg is a bifunctional enzyme able to detect oxidized purines (mainly 8-oxoguanines) and imidazole ring-opened purines (or formamidopyrimidines-Fapy-) (Boiteux et al., 1990, 1992). However the Fpg-modified comet assay detects DNA lesions induced by the alkylating agent MMS; this is due to the conversion of alkylated lesions into ring-opened purines during the lysis, which is performed at pH 10 (Speit et al., 2004; Azqueta et al., 2013a; Hansen et al., 2018). This was demonstrated by Hansen et al. (2018) by comparing the results obtained when using the Fpg-modified comet assay on MMS-treated human lymphocytes with lysis at pH 7.5 and pH 10. Fpg-sensitive sites were concentration-dependent when performing lysis at pH 10, but no Fpg-sensitive sites were detected when the lysis step was performed at pH 7.5. This effect was not seen when using the photosensitizer Ro 19-8022 plus light to induce 8-oxoguanines, as the same levels of Fpg-sensitive sites were obtained at both pHs. This clearly indicates that MMS-induced lesions (alkylated bases) are converted into Fpg-detectable lesions (i.e. ring-opened purines) during the lysis step at high pH and, as Hansen et al. (2018) pointed out, the process is likely to be both pH- and time-dependent. As expected, Fig. 3 shows how Fpg detected $KBrO_3$-induced lesions (i.e. 8-oxo-guanines) and MMS-induced lesions that are converted into ring-opened purines (i.e. N7-methylguanines) in a concentration-dependent manner. The same figures also show how hAAG is not able to detect oxidized bases, but it detects a higher level of alkylated bases than Fpg.

Here we have repeated the experiments performed by Hansen et al. (2018), comparing the performance of the Fpg- and the hAAG-modified comet assays using lysis solution at pH 7 and 10. In this work we used untreated, MMS- and $KBrO_3$- treated TK-6 cells. Results obtained when using the Fpg-modified comet assay are the same as the ones obtained by Hansen et al. (2018): MMS-induced lesions were not detected at pH 7 but detected at pH 10, while no differences were observed in the $KBrO_3$-treated cells (Fig. 4). However, the levels of hAAG-sensitive sites detected were the same independently of the pH of the lysis solution. This demonstrates that hAAG is detecting either lesions that are stable at pH 10 (so it is not detecting N7-methylguanines at all) or it is also detecting ring-opened purines. Considering all the data, the most plausible hypothesis is that hAAG is detecting N7-methylguanines, the ring-opened purines derived at high pH, and the 3-methyladenines, not detected by the Fpg-modified comet assay. (It is worth mentioning that 7-methylguanine occurs at much higher frequency than other alkylated bases (Beranek, 1990)). Clearly, hAAG does not detect 8-oxoguanines.

Figs. 3 and 4 show the results obtained when using the Fpg- and the hAAG-modified comet assay in untreated, MMS- and $KBrO_3$-treated cells in two laboratories using a similar protocol but different formats (12 minigels/slide and Gelbond® films respectively). Experiments were performed using the same batch of cells and same operator (data presented in Fig. 3 were obtained in one laboratory and data presented in Fig. 4 in the other one). hAAG was the same in both laboratories, and it was titrated in each of the laboratories, whereas Fpg was different for each laboratory (both were crude extracts of *E. coli* strains overproducing Fpg but coming from different batches). Fpg is commonly used in both laboratories and it was titrated before this work was carried out (data not shown). As can be observed, results obtained when performing the Fpg- and hAAG-modified comet assay in $KBrO_3$-treated cells are quite similar. However, differences arise when using similar concentrations of MMS. In the case of the Fpg-sensitive sites, it should be noted that the basal level in non-treated cells is very different in both laboratories, from non-detected lesions (Fig. 3) to 10–15 % DNA in tail, corresponding to the net Fpg-sensitive sites (Fig. 4); so detected net Fpg-sensitive sites induced by $KBrO_3$ are a bit lower in the case of Fig. 4 compared with Fig. 3, though the crude values are very similar. In the case of hAAG-sensitive sites, there is a considerable difference between both laboratories; basal levels are similar (non-detected lesions in both laboratories) but there is a huge difference in the levels detected in MMS-treated cells. It seems that this is not due to the difference in hAAG concentration used after the titration experiment (10 U/mL of hAAG in the 12 minigels/slide format -Fig. 3- and 1 U/mL of hAAG in the HTP -Fig. 4-). As can be observed in Fig. 4, there is enough enzyme to detect at least 60 % of net hAAG-sensitive sites (detected in the 10 μM MMS-treated cells) and it only detects about 40 % in cells treated with 5 μM MMS. Most likely, the cell treatment may differ from one laboratory to the other. In any case, the level of hAAG-sensitive sites is higher than the Fpg-sensitive sites in MMS treated cells in all cases.

The objective of the lysis is to remove all the soluble components of the cells and leave naked DNA in form of nucleoids (supercoiled DNA). Detergent (i.e, Triton X-100) included in the lysis solution breaks the membranes, both the cellular and the nuclear, while the high concentration of salts (i.e., NaCl at > 2 M) removes the proteins, including the histones. High pH may help in destabilizing all the cellular component, which is not really needed. However, high pH converts some non-detectable DNA lesions (in terms of comet assay) into others that are detected; on one hand making the assay more sensitive and, on the other, making more difficult the interpretation of the results in mechanistic studies (in which the primary lesions are wanted to be detected). According to results obtained in this work (Fig. 4) and in Hansen et al., 2018, it seems that performing the lysis at neutral pH gives reliable results in terms of Fpg-sensitive sites as oxidized lesions, hAAG-sensitive sites as alkylated lesions and SB; although more studies are needed in this direction. In any case, more experiments are needed to study the effect of high pH of lysis on other DNA lesions.

As mentioned in the introduction, N-alkylations are seen as being mainly cytotoxic, as they are relatively less mutagenic than O-alkylations (Kondo et al., 2010). However, here we have observed the induction of N-alkylations at non-cytotoxic concentrations. A high level of hAAG-sensitive sites, reaching the saturation level of the comet assay, was detected at 5 and 10 μM MMS (Fig. 3). These concentrations induce a RSG between 90 and 100 % (data not show). (At 20 μM MMS the RSG was close to 80 %.) Moreover MMS, which as mentioned before induces N-alkylations, is positive in several mutagenicity assays (Kirkland et al., 2016).

It is worth mentioning that hAAG is also able to detect non-alkylated lesions such as deaminated purine lesions (i.e., hypoxanthine and xanthine) and the lipid peroxidation-derived adduct 1,N6-ethenoadenine (Lee et al., 2009; Taylor et al., 2018). The deamination of bases is of great interest, as there is evidence of the mutation potential of hypoxanthine (DeVito et al., 2017). Regarding 1,N6-ethenoadenine, it can be produced either endogenously (i.e., by lipid peroxidation and/or reactive oxygen and nitrogen species) or by the metabolism of xenobiotics (e.g., vinyl chloride). Etheno-DNA adducts may have a causal role in the initiation and progression of carcinogenesis, as ethenobases produce mainly pair substitution mutations (Nair et al., 1999; Kennedy et al., 2019). To explore this possibility, the incision capability of hAAG towards these and other oxidized DNA lesions was studied using the Glyco-SPOT assay. In this work we have observed that hAAG + Endo III cleaved ethenoadenine, thymine glycol and hypoxanthine at the highest concentration tested (Fig. 5). (The cleavage of thymine glycol was due to the activity of Endo III and not to hAAG - data not shown.) hAAG + Endo III were not able to detect the other lesions included in the Glyco-SPOT assay (i.e., A or C paired with 8oxoG, abasic sites, and uracil paired with G or A).

It should be noted that in the comet assay it is not necessary to

complement hAAG with Endo III or any other enzyme with AP-endonuclease activity for the cleavage. This is explained because in the comet assay, after enzyme incubation the unwinding step is performed at pH > 13. Thus, alkaline conditions allow the cleavage of the AP-sites generated during hAAG incubation into SB, therefore emulating AP-endonuclease activity.

Ethenoadenines and hypoxanthine may also be detected by the hAAG-modified comet assay and this should be taken into account when interpreting the results. Nair et al. (1999) elucidated the effect of dietary fat intake on endogenous DNA damage and the protective effect of antioxidants in terms of production of ethenoadenines by lipid peroxidation. Thus, the use of hAAG in combination with the comet assay in human biomonitoring studies may have special interest.

In conclusion, we have shown for the first time the use of hAAG enzyme in combination with the comet assay for the detection of alkylation damage to DNA, although other lesions may also be detected, such as hypoxanthine and ethenoadenines. Our results support the application of this enzyme in combination with the comet assay, not only for genotoxicity assessment (*in vitro* and *in vivo*) but also in other fields such as human and environmental biomonitoring and ecotoxicology.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 6

Validation of the *in vitro* comet assay for DNA cross-links and altered bases detection

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Abstract

Mechanistic toxicology relevant to human is increasingly warranted for toxicological risk assessment. Different mechanistic assays are available, such as the comet assay, which detects DNA damage at the level of individual cells. However, the conventional version only detects strand breaks and alkali-labile sites. We have validated two modifications of the *in vitro* assay to generate mechanistic information: 1) use of DNA repair enzymes for detection of oxidized and alkylated bases (i.e., formamidopyrimidine DNA glycosylase, endonuclease III, human 8-oxoguanine DNA glycosylase I and human alkyladenine DNA glycosylase) as well as 2) a modification for detecting cross-links. Seven genotoxicants with different mechanisms of action (potassium bromate, methyl methanesulfonate, ethyl methanesulfonate, hydrogen peroxide, cisplatin, mitomycin C and benzo[a]pyrene diol epoxide), as well as a non-genotoxic compound (dimethyl sulfoxide) and a cytotoxic compound (Triton X-100) were tested on TK-6 cells. We were able to detect with high sensitivity and clearly differentiate oxidizing, alkylating and crosslinking agents. These modifications of the comet assay significantly increase its sensitivity and its specificity towards DNA lesions, providing mechanistic information regarding the type of damage.

1. Introduction

During the last years, the Adverse Outcome Pathway (AOP) concept has arisen as a pragmatic tool in the toxicological evaluation of all kind of chemicals based on a more human relevant mechanistic toxicology. AOPs are conceptual constructs aimed to support risk assessment by understanding the mechanism linking a molecular initiating event (MIE, *e.g.*, binding to an enzyme) with an adverse outcome (AO, *e.g.*, heritable mutations or cancer), through a progression of measurable biological changes, known as key events (KE, *e.g.*, DNA alkylation) (Ankley *et al.*, 2010; Leist *et al.*, 2017). Indeed, KE are considered as relevant factors and potential endpoints in decision-making processes for hazard identification (Leist *et al.*, 2017). Indeed, the Organisation for Economic Co-operation and Development (OECD) supports the AOP concept and has prepared a workplan for its development (OECD, 2020).

In this context, the detection and measurement of KE with reliable tools and methods is of great relevance. Regarding genotoxicity assessment, different assays are available for measuring KE, such as the alkaline comet assay (single cell electrophoresis) which is a method to measure DNA damage levels, particularly strand breaks (SB) and alkali-labile sites (apurinic/apyrimidinic -AP-sites or baseless sugars), at the level of individual cells. Its versatility makes it a widely used technique, as it can be applied to any eukaryotic cell type, including disaggregated tissues from which single cells or nuclear suspensions can be obtained (Vasquez, 2012, Azqueta and Collins, 2013, Jackosn *et al.*, 2013, Asare *et al.*, 2016). Indeed, the technique is used in different scientific fields, such as human and environmental biomonitoring or *in vivo* and *in vitro* genotoxicity testing of chemicals and nanoparticles, among others.

The comet assay is relatively simple, and it was developed on its alkaline version by Singh and colleagues in 1988 (Singh *et al.*, 1988). In brief, cells are embedded in agarose on a microscope slide and lysed with detergent and high salt concentration to remove cell membranes and components leaving a nucleoid, which is the supercoiled DNA. Then, the lysed cells are subjected to alkaline conditions to unwind DNA followed by electrophoresis. If the DNA integrity of a nucleoid is disrupted by a SB, supercoiling is relaxed and part of the DNA will extend due to the electrophoretic force giving a comet-like image when evaluated by fluorescence microscope; whereas if DNA remains intact, supercoiling will be preserved (Collins, 2004).

From the regulatory point of view, the *in vivo* version of the comet assay has been validated, and the OECD published an *in vivo* Mammalian Alkaline Comet Assay Guideline (TG 489) (OECD, 2016). The *in vitro* version of the comet assay does not have an OECD guideline, however, its combination with a 3D skin model was validated in a study lead by Cosmetics Europe with the

support of the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) and is currently in the OECD Test Guideline Programme (OECD TGP) work plan (EURL-ECVAM, 2019). Furthermore, the European Food Safety Authority (EFSA) recommends the use of the *in vitro* comet assay in combination with specific enzymes to detect oxidized bases and also to provide complementary information of the genotoxic mechanisms of action of nanomaterials (EFSA, 2018).

In fact, the standard alkaline comet assay only detects SBs and AP-sites, whereas most DNA damaging agents induce other lesions such as oxidized and alkylated bases, adducts or cross-links. To partly overcome this limitation, the comet assay has been modified including a digestion step after lysis with specific DNA-repair enzymes (DNA glycosylases). These enzymes can detect and remove the damaged base, leaving an AP-site, which is then converted to a SB by an associated AP lyase activity of the enzyme or, if the enzyme lacks this activity, by the alkaline pH of the unwinding solution (Azqueta and Collins, 2013; Muruzabal *et al*., 2020a). The frequency of the net-enzyme sensitive sites is calculated by subtracting the DNA damage level of the nucleoids incubated with the enzyme buffer alone from the DNA damage level of the nucleoids treated with the enzymes (Collins, 2009). Most commonly, this modified version of the comet assay has been applied for the detection of oxidized bases using formamidopyrimidine DNA glycosylase (Fpg), endonuclease III (Endo III) and human 8-oxoguanine DNA glycosylase I (hOGG1) (Olsen *et al*., 2003; Smith *et al*., 2006; Hansen *et al*., 2010; Collins, 2014; Muruzabal *et al*., 2020a). The use of enzymes for the detection of alkylated DNA lesions, such as 3-methyladenine DNA glycosylase II (AlkA), 3-methyladenine DNA glycosylase (AlkD) and human alkyladenine DNA glycosylase (hAAG) has also been reported (Collins *et al*., 2001; Hašplová *et al*., 2012; Muruzabal *et al*., 2020b). We recently published a review of the enzymes that have been used in combination with the comet assay that identified 12 different enzymes used for detecting several lesions, mainly oxidized bases (both purines and pyrimidines), but also alkylated bases, uracil residue and pyrimidine dimers (Muruzabal *et al*., 2020a).

Similarly, although less extensively, the comet assay has also been modified for the detection of cross-links. When DNA contains inter-strand cross-links (ICL) within its structure, the extension during electrophoresis in the comet assay is inhibited as the nucleoid is kept compact, thereby exhibiting the opposite effect compared to SBs, hence migrating less compared to DNA of control cells. Thus, ICLs can be detected either by increasing the duration of the electrophoresis to such an extent that even DNA of non-treated cells migrates considerably, or by treating cells with a second genotoxic agent (chemical or physical) for inducing a known number of DNA

breaks. DNA containing ICL will migrate less compared to DNA of control cells in both approaches (Olive *et al*., 1992; Tice *et al*., 1997, 2000).

The objective of this work is to internally validate the performance of two modified versions of the comet assay for the detection of different genotoxic endpoints. Particularly, four different commercially available enzymes (hAAG, hOGG1, Fpg and Endo III), and a widely used non-commercial crude bacterial extract of Fpg, have been used in combination with the comet assay in the analysis of the DNA lesions induced by compounds with different mechanisms of action (*e.g*., oxidizing and alkylating agents) in order to detect several lesions within a single assay. Additionally, cells treated with these compounds were also analyzed employing the comet assay modified for the detection of cross-links. Moreover, the activity of the different enzymes employed in this work and their specificity towards a set of different defined DNA lesions were determined by using a multiplex oligonucleotide-cleavage assay (the Glyco-SPOT assay). The compounds employed were potassium bromate ($KBrO_3$, oxidizing compound mainly inducing 8-oxoguanines), methyl methanesulfonate (MMS, monofunctional alkylating agent), ethyl methanesulfonate (EMS, alkylating agent), hydrogen peroxide (H_2O_2, oxidizing compound), cisplatin (CisPt, cross-linking agent), mitomycin C (Mit. C, cross-linking agent), benzo[a]pyrene diol epoxide (BPDE, bulky-adduct inducer), dimethyl sulfoxide (DMSO, as no-genotoxic control) and Triton X-100 (as cytotoxic compound). The final aim of this work is to provide an assay that could be used as a tool to generate mechanistic information in the current regulatory context for chemical risk assessment.

2. Material and methods

2.1. Chemical and reagents

Low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, Bovine serum albumin (BSA), NaOH, KCl, $KBrO_3$, DMSO, MMS, EMS, H_2O_2, CisPt, Mit. C and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. BPDE was purchased from Santa Cruz Biotechnology. DPBS 1x for mixing cell suspensions with agarose was purchased from Gibco. DPBS without Ca^{+2} and Mg^{+2} 10x from Lonza was used to prepare PBS 1x washing solutions for comet assay slides. Dimethyl sulfoxide was purchased from PanReac AppliChem. All cell culture reagents were purchased from Gibco.

The enzymes hAAG, Endo III and commercial Fpg were purchased from New England Biolabs (catalog number M0313S, M0268S and M0240S respectively); hOGG1 was purchased from R&D Systems, Biotechne (catalog number 4130-100-EB). Non-commercial Fpg from an over-

producing *E. coli* strain was kindly provided by NorGenoTech AS (Oslo, Norway) that distributes the enzyme on request.

2.2. Cell culture

Human-derived lymphoblastoid TK-6 cell line was obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI medium (Roswell Park Memorial Institute; ref. A10491-01, Gibco) containing D-glucose, HEPES, L-glutamine, sodium bicarbonate and sodium pyruvate and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained as a suspension culture, between 0.2 and 1 x 10^6 cells/mL, in continuous agitation at 37 °C in a humidified atmosphere with 5% CO_2. Cells were maintained in culture for no longer than 2 months.

2.3. Treatment of cells

Table 1 shows the different compounds and concentrations tested, including the solvent used for each compound. Preliminary studies were performed to assess cytotoxicity employing the proliferation assay according to Azqueta *et al*. (2013a) with some modifications. Briefly, cell suspensions were counted before and just after treatment and after 24 and 48 h in culture. Then, total suspension growth (TSG) was calculated for each condition dividing the number of cells after 48 h by the number of cells treated. Relative suspension growth (RSG) was calculated by dividing the TSG from each condition tested by the TSG of the solvent control. The mean and SD for RSG of the triplicate experiments are shown in *Table 1*. Although RSG value at 48 h was the criterion of cytotoxicity, in all cases cells were counted immediately after the treatment to measure the cells loss resulting from contact with the chemical. Three concentrations with a RSG over 80% were selected for the enzyme-modified comet assay experiments. An additional concentration of each compound with RSG values below 80% was employed for the comet assay modified for the detection of cross-links (two additional concentrations in the case of cisplatin) except for H_2O_2, which was not tested using this modification (*Table 1*). Triton X-100 was employed as a cytotoxicity control thus its RSG values were lower: 60% for the lowest concentration (0.03 mM) and RSG < 10% for the highest (0.1 mM).

Table I. List of compounds, CAS numbers, solvents, concentrations employed and their respective RSG.

Compound	CAS number	Solvent*	Concentrations**	RSG
$KBrO_3$	7758-01-2	H_2O	0.313 mM	90
			0.625 mM	85
			1.25 mM	78
			2.5 mM	40
MMS	66-27-3	DMSO	5 µM	98
			10 µM	85
			20 µM	80
			40 µM	58
EMS	62-50-0	DMSO	0.5 µM	90
			5 µM	84
			50 µM	77
			100 µM	40
BPDE	55097-80-8	DMSO	0.025 µM	93
			0.05 µM	83
			0.1 µM	70
			0.2 µM	50
H_2O_2	7722-84-1	PBS	125 µM	92
			250 µM	87
			500 µM	79
CisPt	15663-27-1	H_2O	0.83 µM	100
			1.66 µM	100
			3.33 µM	80
			6.66 µM	39
			13.33 µM	12
Mit. C	50-07-7	Medium	0.006 µM	98
			0.03 µM	95
			0.15 µM	82
			0.3 µM	50
Triton X-100	9002-93-1	H_2O	0.03 mM	60
			0.1 mM	7
DMSO	67-68-5	Medium	1%	100
			2%	95
			4%	83
			8%	80

* The final solvent concentration was 1% in all cases.

**The three lowest concentrations of each compound were employed for the enzyme- and comet assay modified for the detection of ICL. The highest concentration (the two highest in the case of CisPt) of each compound was included for the comet assay modified for ICL detection (except for H_2O_2 and Triton X-100).

Treatments were performed as follows: TK-6 cells were seeded in a 12-well plate (1 mL/well) at $1x10^6$ cells/mL in culture medium containing no serum and treated for 3 h with different non-cytotoxic concentrations of the or their vehicles. Treatments were performed with continuous agitation at 37°C in a humidified atmosphere with 5% CO_2. In the case of H_2O_2 the treatment was performed in phosphate-buffered saline (PBS) at 4°C for 5 min, and cells were then washed and seeded in complete culture medium for 1 h and 45 min to repair the strand breaks and leave only the oxidative lesions (this conditions were used based on preliminary studies, data not shown).

After 3 h of treatment, cells were kept ice-cold to prevent DNA repair and cells were centrifuged and washed twice with PBS. After the second centrifugation cells were resuspended in PBS to a final concentration of $2.5x10^5$ cells/mL. These cells were directly employed for the comet assay (see next section). Each compound was tested thrice in the same conditions.

2.4. Enzyme-modified comet assay

The medium-throughput format of 12 minigels per slide of the comet assay (Shaposhnikov *et al*., 2010) was employed according to Muruzabal *et al*. (2018).

All enzymes were previously titrated as described in Muruzabal *et al*. (2018 and 2020b) using nucleoids containing lesions detected by each enzyme (*i.e*., oxidized purines induced by $KBrO_3$ - 0.313-1.25 mM - for hOGG1 and Fpg; oxidized pyrimidines induced by H_2O_2 - 125-500 µM - for Endo III; alkylated bases induced by MMS - 5-20 µM - for hAAG). The concentration allowing the detection of the maximum level of DNA lesions without inducing non-specific breakage was selected and employed for each enzyme in these experiments.

To mold the minigels, thirty microliters of cell suspension (either treated or non-treated cells, previous section) were mixed with 140 µL of 1% low melting point agarose in PBS at 37°C. Then, 5 µL droplets of the corresponding cell suspension-agarose mix were placed on agarose-precoated slides. The slides were placed on the metal holder of the 12-Gel Comet Assay Unit™ (NorGenoTech, Oslo, Norway), which contains a template to set the minigels in precise positions (2 rows of 6) and was previously cooled in the fridge. Each slide contained 12 minigels of the same suspension so 6 replicates were designed for incubation: one pair of minigels to be incubated with the enzyme reaction buffer and the remaining five pairs to be incubated with each of the enzymes. Therefore, as 3 concentrations and control cells (*i.e*., cells exposed to the compound solvent) were tested with each compound, 4 slides per compound were prepared.

Once slides were prepared, cells were lysed by immersing the slides in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris base, pH 10 and 1% Triton X-100) at 4 °C for 1 h. Then slides were washed three times, 5 min each, at 4°C with the reaction buffer of the enzymes (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/ml BSA, pH 8). During the washes, the enzymes were prepared by diluting original stocks with reaction buffer. It should be noted that the enzyme reaction buffer employed for the washes was also employed for diluting all enzymes to their optimal concentration.

After washing, slides were transferred to a cold 12-Gel Comet Assay Unit™ to incubate each of the gels separately, as gels are isolated as wells on the slides. Units were placed on a cold metal plate to keep them cold during the enzymes or reaction buffer addition to avoid enzymatic reactions until incubation. Thirty microliters of reaction enzyme buffer or of the corresponding enzyme were pipetted to each well and a clean slide was placed on top of the unit to cover all wells and prevent contamination and evaporation. The design of each slide was the same in all cases: 2 minigels were incubated with enzyme buffer alone and each enzyme was used on 2 minigels, thereby completing the remaining 5 pairs of minigels. The 12-Gel Comet Assay Units™ were transferred to a pre-heated moist box and placed in the incubator for 1 h at 37°C.

After incubation, units were placed on a cold plate to stop the enzyme reaction and slides were removed from the chambers and transferred to the electrophoresis tank for unwinding in electrophoresis solution (0.3M NaOH, 1mM Na_2EDTA, pH > 13) for 40 min in a 4°C cold room. Afterwards, electrophoresis was carried out at 1.2 V/cm for 20 min at 4°C.

After electrophoresis, slides were neutralized by washing them in PBS for 10 min and rinsed in distilled water for further 10 min. To dehydrate the gels for avoiding edge effect (*i.e.*, comets going in different angles) (Azqueta *et al*., 2013b), slides were immersed in 70% ethanol for 15 min and then in absolute ethanol for further 15 min to let them dry overnight at room temperature.

Finally, each minigel was stained with a 5 µL drop of 1 µg/mL of 4,6-diamidino- 2-phenylindole (DAPI) and all minigels of the slide were covered using a coverslip (24 x 60 mm). After 30 min of incubation with DAPI at room temperature, slides were analyzed using the semi-automated image analysis system Comet Assay IV (Perceptive Instruments) and 50 nuclei per gel, 100 per condition, were scored. The percentage of DNA in tail (or tail intensity) was used as descriptor for each comet. The median percentage of DNA in tail for 50 comets was calculated for each of the duplicate minigels and the mean of the two medians was taken as the measure of DNA damage for each condition in each of the three independent experiments. Net enzyme-sensitive

sites were calculated by subtracting the percentage tail DNA obtained with the buffer incubation alone from that obtained after incubation with each enzyme. The value of buffer incubation alone was representative of SB and ALS of each sample. Three independent experiments were carried out and results are expressed as the mean of the three experiments ± SD.

2.5. Modification of the comet assay for detecting cross-links

The format of 12 minigels per slide of the comet assay was also employed for this modification. Procedure for molding the gels was the same as previously described. However, prior to the lysis step slides were transferred to the 12-Gel Comet Assay Units™ for treating the gels with H_2O_2 to induce a known amount of DNA damage (*i.e*., around 40-50% of DNA in tail, in terms of tail intensity). Particularly, gels were treated for 5 min at ice-cold temperature with H_2O_2 97.9 mM (100 µL per well) (concentration and conditions stablished according to preliminary experiments) and then washed with cold PBS. Then slides were placed in a Coplin jar for the lysis step. From this point, remaining procedure was the same as in the previous section, but no enzyme or reaction buffer-incubation step was performed with these slides (*i.e*., slides where immersed in the electrophoresis solution for the alkaline treatment after the lysis). All solutions and reagents were the same as explained in section 2.4.

When a reduction in DNA migration was observed in cells treated with a compound, further analysis was performed to quantify the reduction level. To do so, results were normalized using its respective control and the percentage of reduction in DNA migration was obtained by subtracting normalized data of each concentration to the control value.

2.6. Glyco-SPOT assay

The multiplexed oligonucleotide (ODN) cleavage assay on support (*i.e*., Glyco-SPOT assay, LXRepair, La Tronche, France) was used according to Muruzabal *et al*. (2020b) to simultaneously control the activity of the enzymes toward several potential substrate lesions. In brief, the assay consists in 24-wells glass slides (Streptavidin-coated, Xantec bioanalytics, Germany) functionalized with a panel of ODNs bearing different lesions. Particularly, the included lesions were: 8-oxoguanine paired with cytosine (8oxoG-C), adenine paired with 8oxoG (A-8oxoG), ethenoadenine paired with thymine (EthA-T), hypoxanthine paired with thymine (Hx-T), tetrahydrofuran (which is an abasic site stable analog) paired with adenine (THF-A), thymine glycol paired with adenine (Tg-A) and uracil paired either with guanine or with adenine (U-G and U-A respectively). All lesions were labeled with a Cy3 at their end as described in Candéias *et al*. (2010) and Pons *et al*. (2010).

Each ODN was immobilized in duplicated in each well together with a Control-ODN that contained no modification. As hAAG was previously analyzed using this assay (Muruzabal *et al*., 2020b), the procedure was performed with both Fph, hOGG1 and with Endo III. Particularly, five concentrations of each enzyme were tested (Fpg: 0.0002, 0.001, 0.005, 0.01 and 0.05 U/well; Fpg.A: 1:2,000,000, 1:1,000,000, 1:500,000, 1:100,000 and 1:20,000 dilutions from the original stock; hOGG1: 0.00016, 0.0008, 0.004, 0.02 and 0.1 U/well; Endo III: 0.0008, 0.004, 0.02, 0.1 and 0.5 U/well) in two different wells in the same enzyme-reaction buffer employed for the enzyme-modified comet assay (see section 2.4 for details). The excision reaction was run for 60 min at 37°C under agitation (700 rpm). Then the slides were washed 2 x 5 min in PBS containing 0.2 M NaCl - 0.05% tween 20 and dried by centrifugation.

Each spot fluorescence was quantified at 532 nm wavelength using the Innoscan 710AL scanner from Innopsys (Toulouse, France) and the associated MAPIX software. Data were normalized as described using NormalizeIt software (Millau *et al*., 2008). To calculate the final cleavage rate of each ODN-containing lesion, the fluorescence of the control well, incubated with the excision buffer only, was taken as reference (100% fluorescence). The data were also corrected by the control-ODN cleavage rate that remained below 10%. Finally, the lesion-ODN cleavage percentage was 100 x (1 - percentage of fluorescence of lesion-ODN/percentage of fluorescence of control-ODN).

2.7. Statistical analysis

Non-parametric one-way analysis of variance (*i.e*., Kruskal-Wallis test) followed by Bonferroni test was applied to compare the levels of net-enzyme sensitive sites (in terms of % DNA in tail), in the enzyme-modified comet assay, and the levels of SBs, in the assay modified for the detection of cross-links, obtained in TK-6 cells treated with different compounds with their respective vehicle controls. Differences showing p value < 0.05 were considered statistically significant. Analyses were carried out using STATA v.12.0 software package.

3. Results

In preliminary experiments, we measured RSG using the proliferation assay with a broad range of concentrations of each chemicals to perform the final assays with non-cytotoxic (RSG 48h after the treatment > 80%) or mildly cytotoxic concentration (for the comet assay modified for ICL detection an additional concentration with RSG < 80%) (*Table 1*).

3.1. Enzyme-modified comet assay

Figure 1 shows the results from the nucleoids treated with the enzyme buffer and the net-enzyme sensitive site of each enzyme enzymes. In all conditions tested (*i.e*., treated, and non-treated cells) with all compounds, the level of DNA damage (in terms of % tail intensity) obtained with the enzyme buffer ("Buffer") was below 5% (*Figure 1a-i*), indicating that very low levels of SBs were induced at concentrations employed with the tested chemicals. Furthermore, after analyzing "Buffer" values of each compound, no concentration-related effects were observed.

The cells treated with $KBrO_3$ (*Figure 1a*), an oxidizing compound (induces mainly 8-oxoguanines), showed a very similar response between non-commercial and commercial Fpg enzymes ("Fpg.A" and "Fpg" respectively). A highly significant increase of both Fpg-sensitive sites depending on the $KBrO_3$ concentration was found compared to non-treated cells. Indeed, highly significant differences ($p < 0.001$) were found in all $KBrO_3$ concentrations tested compared to non-treated cells (*Fig. 1a*). hOGG1 response was similar to the one obtained with both Fpg enzymes, but the levels of hOGG1-sensitive sites were slightly lower at the lowest $KBrO_3$ concentration. When comparing hOGG1-sensitive sites in treated versus non-treated cells, significant differences were found from 0.625 mM of $KBrO_3$ onwards. hAAG and Endo III did not show any activity in $KBrO_3$-treated cells, and no increase in hAAG- or Endo III-sensitive sites was detected compared to non-treated cells at any of the tested concentrations.

MMS, a monofunctional alkylating agent, also induced a similar response in treated cells when analyzed with both Fpg enzymes (*Fig. 1b*). A concentration-dependent increase in enzyme-sensitive sites was observed with both Fpg enzymes, being significant from 5 µM onwards in the case of commercial Fpg, and from 10 µM onwards in the case of non-commercial Fpg. Regarding hAAG, the increase in enzyme-sensitive sites in treated cells was highly significant ($p < 0.001$) compared to non-treated cells from the lowest concentration onwards, and more sharpened compared to both Fpg. The response observed with hOGG1 and Endo III also showed an MMS concentration-dependent increase, but in a more limited way, as the enzyme-sensitive sites were considerably lower compared to the other enzymes; significant differences ($p < 0.05$) compared to non-treated cells were only found at 20 µM MMS (*Fig. 1b*).

EMS is another alkylating agent inducing ethyl groups, and its effects on treated cells (in terms of enzyme-sensitive sites) were only noticeable at the highest concentration (*i.e*., 50 µM) with hAAG (*Fig. 1c*). Indeed, the level of hAAG-sensitive sites in cells treated with 50 µM of EMS was significantly higher compared to levels detected in non-treated cells ($p < 0.001$). Regarding the other enzymes tested, no activity was detected at any of EMS concentrations tested (*Fig. 1c*).

H_2O_2 induces both, oxidized DNA lesions and SB in DNA of treated cells. To study base damage specifically, cells were incubated after treatment to repair SB leaving only oxidative DNA damage. Both Fpg enzymes showed a similar pattern in detecting H_2O_2-induced lesions in TK-6 cells, showing a concentration-dependent increase in enzyme-sensitive sites (*Fig. 1d*). Indeed, significant differences compared to non-treated cells were found at 250 and 500 µM H_2O_2 with Fpg.A ($p < 0.05$ and $p < 0.01$ respectively) and at 500 µM with Fpg ($p < 0.01$). The pattern of response observed with hOGG1 was similar to the observed with both Fpg enzymes but with lower tail intensities. A significant increase in DNA damage was only detected at the highest H_2O_2 concentration (*i.e.*, 500 µM) compared to non-treated cells ($p < 0.05$). Regarding Endo III a small but significant increase in enzyme-sensitive sites was found at the highest concentration of of H_2O_2 (500 µM) ($p < 0.05$). No response was observed with hAAG at any of H_2O_2 concentrations tested (*Fig. 1d*).

No activity was detected for any enzyme at any of the tested concentrations of CisPt (cross-linking agent) (*Fig. 1e*), BPDE (bulky adducts inducer) (*Fig. 1f*) and Mit. C (cross-linking agent) (*Fig. 1g*). Finally, regarding Triton X-100 and DMSO, the non-genotoxic compounds employed, no increase in DNA damage was detected at any of the tested concentrations with any of the enzymes (*Fig. 1h* and *i* respectively). Overall, no statistically significant differences were found for the Fpg enzymes with any of the tested compounds.

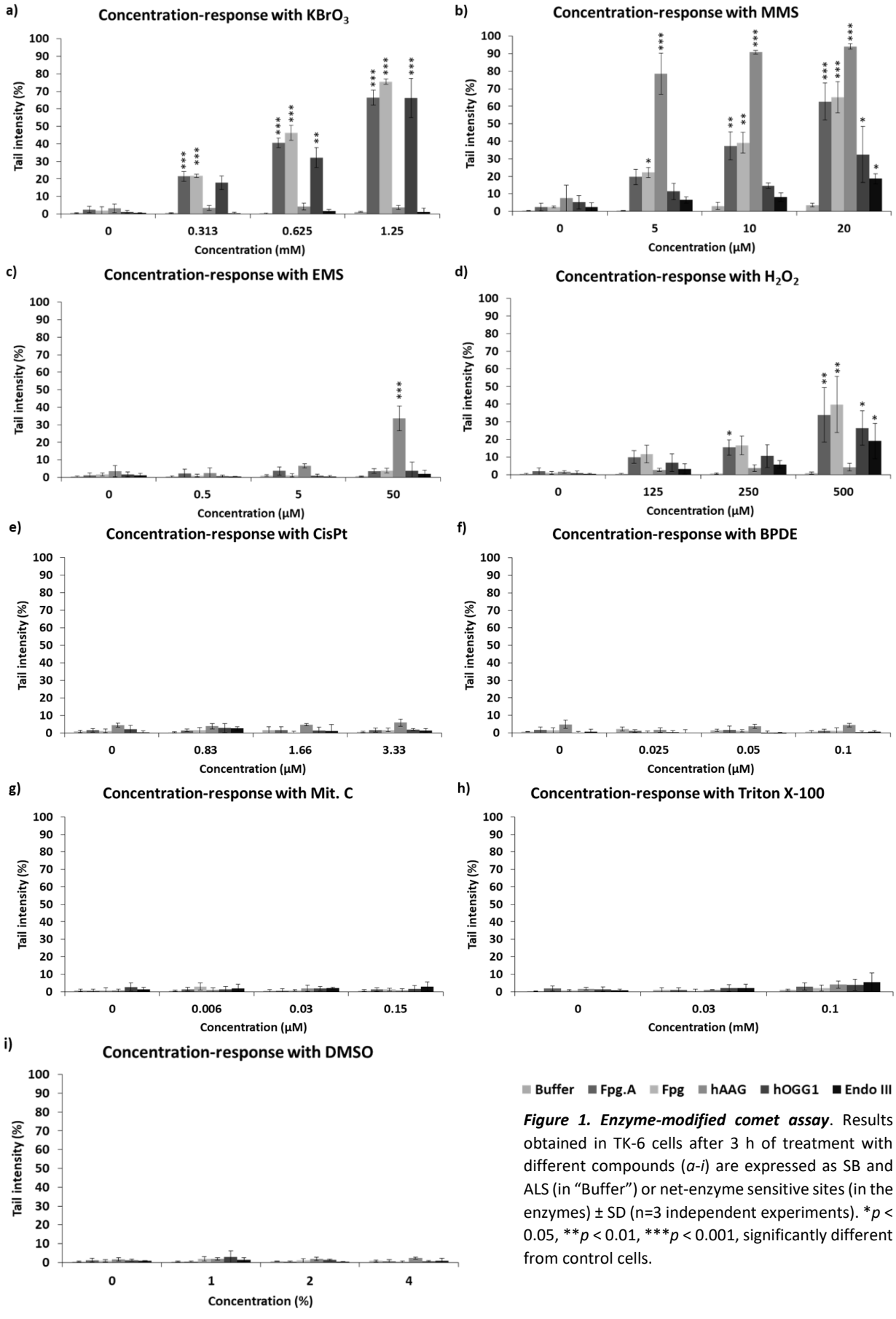

Figure 1. Enzyme-modified comet assay. Results obtained in TK-6 cells after 3 h of treatment with different compounds (*a-i*) are expressed as SB and ALS (in "Buffer") or net-enzyme sensitive sites (in the enzymes) ± SD (n=3 independent experiments). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, significantly different from control cells.

3.2. Modification of the comet assay for detecting cross-links

Compared to the enzyme-modified comet assay, an additional higher concentration of each compound with RSG levels < 80% was tested. For the detection of cross-links, in addition to the treatment with each compound, a second treatment was performed (once comet assay gels were molded on the slides) with H_2O_2 to induce about 40-50% of DNA damage (in terms of tail intensity).

In cells treated with CisPt, a concentration-dependent decrease in the levels of H_2O_2-induced DNA damage was observed, with 43 ± 5.3% of DNA in tail in non-treated cells and 27 ± 3.7% of DNA in tail at the highest CisPt concentration, representing a reduction of 35 ± 15.5% of DNA migration. Indeed, this reduction was significant ($p < 0.05$) and highly significant ($p < 0.01$) at 6.66 and 13.33 µM of CisPt respectively (*Fig. 2d*). Mit.C also induced a concentration-dependent decrease in H_2O_2-induced DNA damage (*Fig. 2f*). This reduction was especially pronounced at the highest Mit.C concentrations tested, in which tail intensity levels were reduced from 41.5 ± 3.5% in non-treated cells to 31 ± 5.2% and 27 ± 7% in 0.15 and 0.3 µM respectively, which represent a reduction of DNA migration of 23 ± 12.2% and 33 ± 13.3% respectively. In the case of the highest concentration (*i.e.*, 0.3 µM) of Mit.C, the reduction in detected DNA damage was significantly reduced compared to non-treated cells ($p < 0.05$).

Regarding cells treated with the other genotoxic compounds ($KBrO_3$, MMS, EMS and BPDE) (*Fig. 2a*, *b, c* and *e*) the H_2O_2-induced DNA damage did not show neither an increase nor a decrease with any of the compounds tested. Indeed, DNA damage values (in terms of tail intensity) found with all concentrations in all compounds remained at 40-50% of DNA in tail with no significant variations (*Fig. 2a, b, c* and *e*). Similarly, cells treated with non-genotoxic compounds (Triton X-100 and DMSO) (*Fig. 2g* and *h*) did not show any significant variation in detected DNA damage, which in all cases were 40-50%. However, Triton X-100 seemed to induce a slight concentration-dependent increase in DNA damage, although it was not significant compared to control cells (*Fig. 2h*).

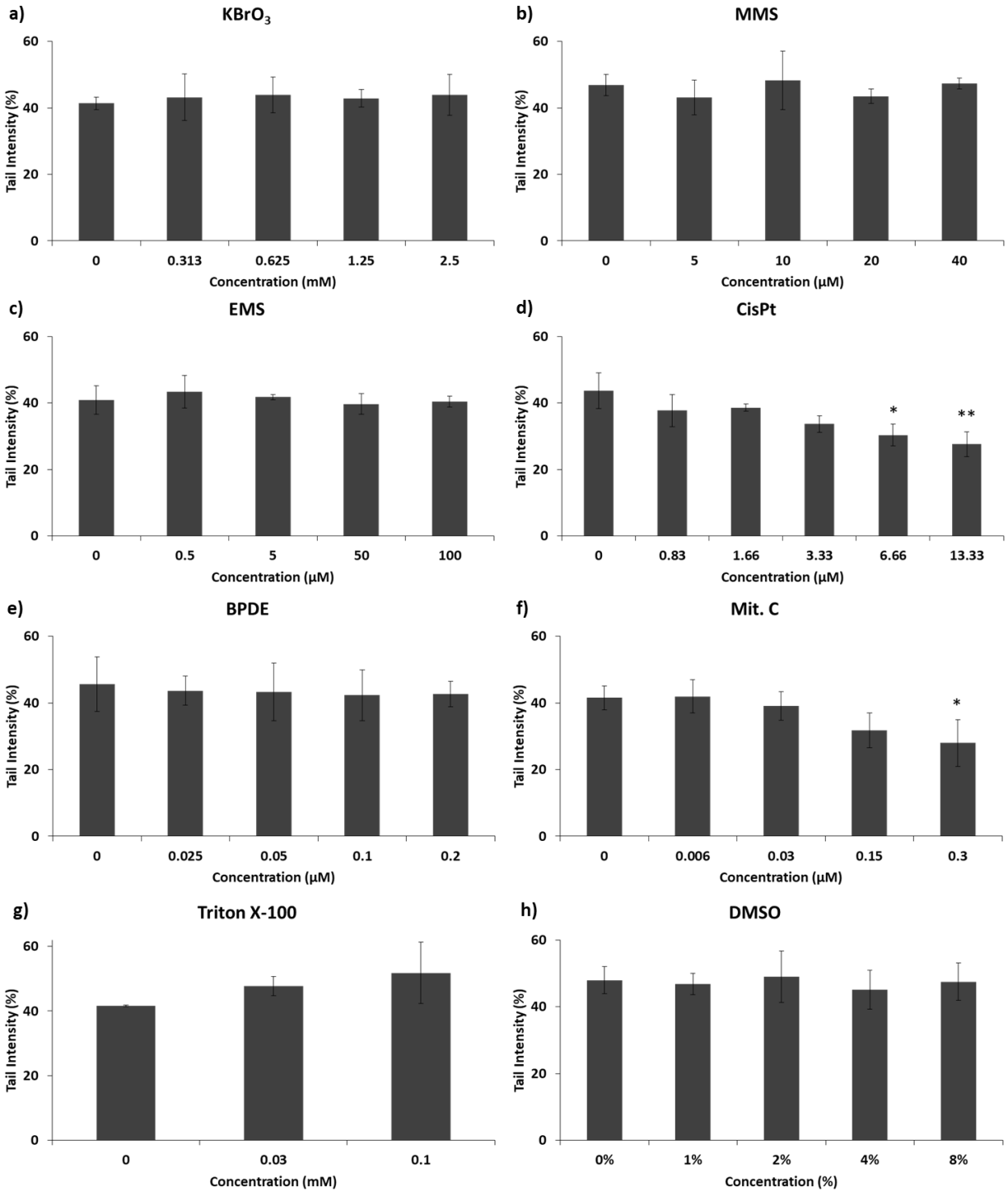

Figure 2. Modification of the comet assay for cross-links detection. Figures show results obtained with TK-6 cells treated for 3 h with different compounds (a-h) and treated again (once molded for the comet assay) with H_2O_2 to induce around 40-50% of DNA in tail (*i.e.,* DNA damage). Mean of SB ± SD (n=3 independent experiments) are represented. Reduction of tail migration in terms of tail intensity indicate the presence of cross-links. *$p < 0.05$, **$p < 0.01$, significantly different from control cells.

3.3. Glyco-SPOT assay

Incubation of the different lesions with increasing concentrations of Fpg, Fpg.A and hOGG1 resulted essentially in the cleavage of 8-oxoguanine paired with cytosine (8oxoG-C) (*Figure 3 a-c*), while Endo III cleavage thymine glycol paired with adenine (Tg-A) (*Figure 3d*). (Results regarding hAAG are already published (Muruzabal *et al*., 2020b)).

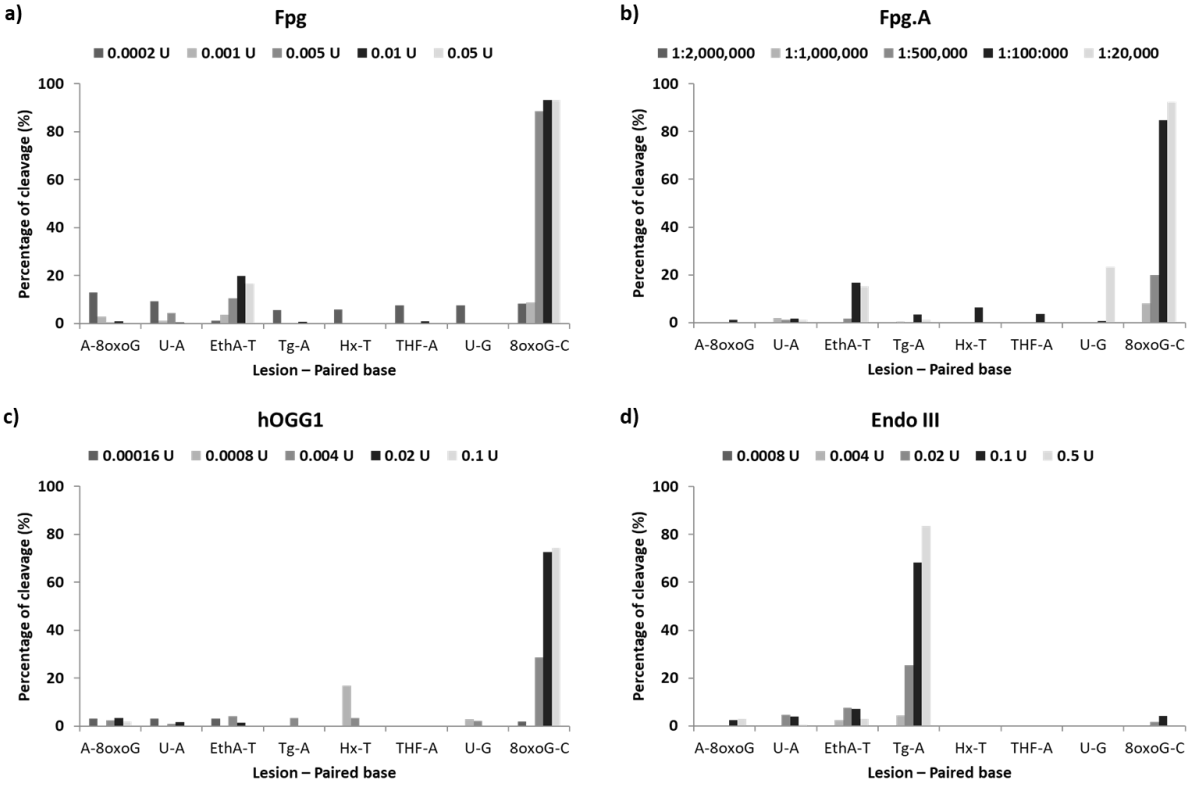

Figure 3. Normalized percentage of cleavage induced by the enzymes in the different DNA lesions included in the Glyco-SPOT assay. Figures show results obtained with commercial Fpg (a), non-commercial Fpg (Fpg.A) (b), hOGG1 (c) and Endo III (d). A-8oxoG: A paired with 8oxoG; 8oxoG-C: 8oxoG paired with C; Hx-T: hypoxanthine paired with T; EthA-T: ethenoadenine paired with T; Tg-A: thymine glycol paired with A; THF-A: tetrahydrofuran -abasic site stable analog- paired with A; U-G: uracil paired with G; and U-A: uracil paired with A.

4. Discussion

The study of mechanisms of action and its potential linkage with AO is gaining relevance in toxicological evaluation in a regulatory context. For this reason, developing tools for the detection of the KE involved in AOPs becomes essential for the development of this mechanistic approach for risk assessment. The inclusion of an *in vitro* comet with its mechanistic modifications is in line with the strategic inclusion of so-called New Approach Methodologies (NAMs) in human risk assessment (Parish *et al*., 2020)

In this study we evaluated the sensitivity of two modifications of the comet assay for detecting an extended variety of DNA lesions induced by compounds with different mechanisms of action. Regarding the use of enzymes, we employed different enzymes for the detection of oxidized bases (hOGG1 and Fpg for oxidized purines and Endo III for oxidized pyrimidines) as well as alkylated bases (using hAAG). We also included a non-commercial Fpg enzyme preparation (from an over-producing *E. coli* strain), which is widely employed among comet assay users, to compare its performance with a commercial Fpg. *Figure 1a-i* shows DNA damage levels obtained when employing the different enzymes in cells treated with several compounds (seven *in vitro* genotoxicants with different mechanisms of action plus two non-genotoxic compounds) and in non-treated cells. In preliminary studies, all enzymes were titrated according to Muruzabal *et al*. (2018 and 2020b) (data not shown) using the same reaction buffer.

As previously explained, when performing the enzyme-modified comet assay, all compounds were evaluated at non-cytotoxic concentrations (RSG>80%, Table 1) to reduce the chance of false positive results, as acute toxic effects may induce DNA damage detected in the Comet assay (Henderson *et al*., 1998). Moreover, we included Triton X-100 as non-genotoxic but cytotoxic compound, at a medium (75% RSG) and high cytotoxic (RSG < 10%) concentrations. Interestingly, under our conditions negative results would be obtained with the standard comet assay with the genotoxic compounds, as results obtained with the enzyme buffer alone did not show DNA damage, which suggest that no SBs were induced under the conditions tested. This indicates that the enzyme-modified comet assay significantly increases not only the sensitivity of the assay but also its specificity, providing mechanistic information about the type of damage.

To date, Fpg is one of the most used DNA repair enzymes in combination with the enzyme-modified comet assay (Muruzabal *et al*., 2020a). Fpg is a bacterial enzyme able to detect 8-oxoguanine, other purine oxidation products, and ring-opened purines (also known as formamidopyrimidines -Fapy-). For this reason Fpg also detects N7-guanine adducts indirectly formed from alkylated bases, as these lesions turn into Fapy due to the high pH of the lysis solution in the comet assay (Dusinska and Collins, 1996; Speit *et al*., 2004; Hansen *et al*., 2018; Muruzabal *et al*., 2020b). We compared the performance of the commercial Fpg and the non-commercial bacterial Fpg extract (from an overproducing *E. coli* strain), both of them widely used in the comet assay, was the same towards DNA lesions induced by several compounds. Both enzymes showed similar activity toward the compounds at identical concentrations, where the maximum levels of variations between the enzymes were around 5% in terms of enzyme-sensitive sites (*Fig. 1a-i*). The higher level of enzyme-sensitive sites was found with $KBrO_3$ (*Fig. 1a*), which specifically induces oxidized purines (mainly 8-oxoguanines) with little or no

induction of SBs (Møller *et al*., 2015), and with MMS (*Fig. 1b*), a monofunctional alkylating agent. As previously reported, results with both compounds were as expected, in the case of MMS due to the aforementioned conversion of N7-alkylated adducts into Fapy, which are detectable by Fpg (Speit *et al*., 2004; Hansen *et al*., 2018; Muruzabal *et al*., 2020b). The treatment with H_2O_2 also induced Fpg-sensitive sites (*Fig. 1d*), although to a much lesser extent compared to $KBrO_3$ and MMS. For the other compounds tested, no increases in DNA damage levels were detected by the Fpg enzymes under our test conditions. When we analyzed the activity and specificity for DNA lesions of the Fpg enzymes using the Glyco-SPOT assay (*Figure 3a* and *b*), similar response was obtained, as cleavage was only detected for 8-oxoguanine paired with cytosine.

hOGG1 is the eukaryotic functional homologue of Fpg. This glycosylase initiates excision of oxidized purines (mainly 8-oxoguanine paired with cytosine) (Bjørås *et al*., 1997; Radicella *et al*., 1997), although it has been reported that is also able to detect Fapy guanines (Bjørås *et al*., 1997; David and Williams, 1998; Lukina *et al*., 2013). The DNA lesions levels detected using hOGG1 with the oxidizing agents, $KBrO_3$ and H_2O_2, were very similar to that observed with Fpg, but the levels of hOGG1-sensitive sites were lower in the case of H_2O_2 (*Fig. 1a* and *d*). Additionally, hOGG1-sensitive sites were detected in MMS-treated cells, mainly at the highest concentration tested (*Fig. 1b*). This is coherent with its ability to detect Fapy-guanines (Bjørås *et al*., 1997; David and Williams, 1998; Lukina *et al*., 2013). One plausible explanation is that, under our conditions, the hOGG1 affinity for Fapy lesions (derived from the transformation of alkylated bases to ring-opened purines due to the alkaline conditions of the comet assay) is lower than for both Fpg enzymes, as the detected levels with hOGG1 were considerably lower. Alternatively, we can also hypothesize that the high concentrations of MMS used herein may be indirectly inducing oxidized damage in vitro (Mizumoto *et al*., 1993). Such DNA lesions are detectable by hOGG1 and Fpg, and the difference in enzyme-sensitive sites may correspond to a higher affinity for Fapy lesions in the case of Fpg, that are theoretically induced in a higher amount. It is also possible that hOGG1 detects other oxidized lesions, indirectly induced by MMS, that is not detected by Fpg, and that Fapy lesions are either detected with lower affinity or not detected by hOGG1, thereby explaining the differences observed. As expected, hOGG1 did not show any activity with the other compounds tested, either genotoxic or non-genotoxic. Regarding Glyco-SPOT assay for evaluating hOGG1 specificity, substrate specificity was similar as with Fpg enzymes, (8-oxoguanine paired with cytosine (*Figure 3c*)).

Endo III is a bacterial glycosylase involved in the excision of a wide range of oxidized pyrimidines, including thymine glycol, 5-hydroxycytosine or cytosine glycol (Doetsch and Cunningham, 1990; David and Williams, 1998). Endo III-sensitive sites were detected in cells treated with H_2O_2 and

MMS. Results obtained with H_2O_2, although expected and significantly higher compared to non-treated cells, were low in terms of enzyme-sensitive sites when compared with hOGG1 or Fpg (*Fig. 1d*). A plausible explanation is that oxidized lesions induced by H_2O_2 may be preferentially located on purine bases, as guanine bases exhibit the lowest ionization potential among DNA bases (Cadet *et al*., 2014). As with hOGG1, although in a lesser extent, Endo III-sensitive sites were detectable in cells exposed to MMS, especially at higher concentrations (*Fig. 1b*). It has been reported that Endo III can detect Fapy adenines (Dizdaroglu *et al*., 2000) which may explain Endo III-sensitive sites obtained in MMS-treated cells. Indeed, as MMS induces alkylated adenines in a lesser extent compared to alkylated guanines (Beranek, 1990), the number of these lesions transformed into Fapy adenines due to the alkaline pH in the comet assay is lower compared to Fapy guanines. Alternatively, similarly as aforementioned with hOGG1, MMS may indirectly induce oxidative DNA damage, which is detected by Endo III. Interestingly, no Endo III-sensitive DNA lesions were observed with $KBrO_3$, indicating that no oxidized purines were detected with Endo III. Finally, no response was observed with other genotoxic and non-genotoxic compounds tested. As expected, Endo III only showed specific activity towards thymine glycol paired with adenine in the Glyco-SPOT assay (*Figure 3d*).

Smith *et al*. (2006) compared the substrate specificity of Fpg, Endo III and hOGG1 in the enzyme-modified comet assay by using $KBrO_3$ (to induce DNA oxidation) and MMS (to induce DNA alkylation) in mouse lymphoma cells. In a different cell line, their results with $KBrO_3$ when using Fpg and hOGG1 were largely similar to ours. However, they detected $KBrO_3$-derived lesions with Endo III, especially at the highest concentration tested with a RSG of 39%, which is a level of toxicity higher than in our study. Regarding results with MMS, they also observed a high response with Fpg at all concentrations tested. Interestingly, they observed a concentration-dependent increase in Endo III-sensitive sites in MMS-treated cells in a much greater extent compared to our results. As they also observed Endo III-sensitive sites after treating the cells with $KBrO_3$, we speculate that these differences may be due to the differences in cellular response to the genotoxicant in the two cell lines. Alternatively, the non-commercial enzyme batch of Endo III, was not as specific as the commercial Endo III used in our study (*Figure 3d*). Indeed, residual nucleases may induce non-specific enzymatic activity in the crude bacterial extracts when enzyme purification is not complete (observation from the authors).

Unlike our results, Smith and colleagues (2006) did not detect hOGG1-sensitive sites in MMS-treated cells, whereas we measured significant increases in hOGG1-sensitive sites at the highest MMS concentration (*i.e*., 20 µM) for which RSG value was 80%, as compared to the highest concentration tested in their study (*i.e*., 22.7 µM) with RSG value of 92%. This difference in

cytotoxicity combined with the fact that different cell lines are being employed may partially explain these differences. However, according to hOGG1-manufacturer data, we should expect some activity of hOGG1 towards Fapy lesions that, presumably, are present in MMS-treated cells as a result of the conversion of alkylated bases due to the alkaline conditions of the comet assay.

Recently, we described the use of hAAG in combination with the comet assay for the first time (Muruzabal *et al*., 2020b). This enzyme is a monofunctional glycosylase responsible of initiating the base excision repair (BER) pathway for repair alkylated bases. Particularly, the enzyme detects 3-methyladenine and 7-methylguanine (O'Connor, 1993) as well as other non-alkylated lesions including deaminated purine lesions (*i.e*., hypoxanthine and xanthine) and the lipid peroxidation-derived adduct 1,N6-ethenoadenine (Lee *et al*., 2009; Taylor *et al*., 2018). Thus, as expected, hAAG-sensitive sites were only found in cells treated with alkylating agents (*i.e*., MMS and EMS) (*Fig. 1b* and *c* respectively) although with different levels of sensitivity. Indeed, MMS-induced lesions were detected by hAAG enzyme with high sensitivity from the lowest MMS concentration onwards (*Fig. 1b*), whereas EMS-induced lesions were only revealed at the highest EMS concentration tested (*Fig. 1c*). This may be explained because despite both agents alkylate purine bases, MMS induces 3-methyladenines and 7-methylguanines and EMS induces 3-ethyladenines and 7-ethylguanines (Beranek, 1990). Interestingly, no response was observed with the other compounds. We previously analyzed the specificity of hAAG using the Glyco-SPOT assay, demonstrating its activity for ethenoadenines and hypoxanthine, but we could not test the enzyme with alkylated DNA lesions since they are not included in the assay (Muruzabal *et al*., 2020b).

Overall, no enzyme detected lesions induced by neither cross-linking agents (CisPt and Mit.C) nor bulky adducts induced by BPDE. Regarding cross-links, we modified the comet assay for its detection by inducing similar levels of fragmentation (SBs) of DNA in all samples (after the respective compound treatments) by exposure to H_2O_2 to establish a known level of DNA damage (*i.e*., approximately 40-50% of DNA in tail). Thus, when cross-links are present in DNA, a retardation in DNA migration is caused and comet tails will appear shorter compared to control samples, that will show the expected amount of DNA damage. In this study, we were able to specifically detect the effects of the two cross-linking agents employed, CisPt and Mit. C (*Fig. 2d* and *f* respectively). CisPt induces mainly intra-strand cross-links, but also ICLs and DNA-protein cross-links (Zamble and Lippard, 1995; Sanderson *et al*., 1996); and Mit. C induces ICLs (Tomasz, 1994). It should be noted that the significant decrease in the number of SBs found after treatments with 6.66 and 13.33 µM of CisPt and 0.3 µM of Mit. C were obtained at cytotoxic concentrations (RSG of 39, 12 and 50% respectively). A non-significant decrease was already

detectable at non-cytotoxic concentrations with both compounds. As expected, no effect was observed with the genotoxic compounds with other mechanisms of action or with the non-genotoxic compounds, although we found a slight and non-significant concentration-dependent increase in SBs levels of cells treated with Triton X-100, especially at the highest concentration tested (0.1 mM) which is probably due to the high toxicity levels (RSG of 7%) (*Figure 2g*).

As aforementioned, we did not detect DNA adducts induced by BPDE with any of the modifications employed in this study. BPDE is the ultimate and DNA reactive metabolite of benzo[a]pyrene (B(a)P) and responsible of B(a)P carcinogenesis (reviewed in Shimada, 2006). Interestingly, Azqueta and colleagues (2013a), reported that the Fpg-modified comet assay increased the sensitivity of the assay towards B(a)P-derived lesions in TK-6 after bioactivation with S9 fraction. This can be explained as it has been shown that during B(a)P metabolism reactive oxygen species may be formed (Flowers *et al*., 1997), detectable by Fpg. When the metabolite BPDE alone is used, it is expected to selectively induce bulky adducts in DNA without inducing radical oxygen species, and thus no DNA damage is expected to be detected with the Fpg-modified comet assay. The bacterial enzyme uvrABC, responsible for nucleotide excision repair system (NER) in prokaryotes is active towards a wide range of substrates including bulky DNA adducts (Sancar and Sancar, 1988), and it was employed in combination with the comet assay (Dušinská and Collins, 1996), but has not given satisfactory results. However, it is possible to detect these lesions by combining the comet assay with the use of DNA repair inhibitors, such as aphidicolin, hydroxyurea and 1-β-Darabinofuranosylcytosine (Gedik *et al*., 1992; Martin *et al*., 1999; Jansen *et al*., 2001; Speit *et al*., 2004; Güerci *et al*., 2009; Vande Loock *et al*., 2010); although some authors recently hypothesize that this method may also inhibit BER intermediates, thereby reducing the sensitivity of the assay (Ngo *et al*., 2020).

The use of the same enzyme reaction buffer along with the medium throughput format of 12 minigels/slide format of the comet assay facilitated the screening of different lesions in a single assay, as each experiment was much less time-consuming. Although both modifications employed (enzymes and cross-links detection) were performed sequentially, it is completely reasonable to integrate the modifications on a single experiment by including an extra slide in each experiment for the second treatment with H_2O_2 for cross-links detection. Indeed, we have already prepared and successfully applied a protocol including both modifications within a single-integrated experiment.

Overall, we specifically detected oxidized and alkylated bases, and cross-links by including different DNA glycosylases in the comet assay as well as the comet assay modified for cross-links

detection, respectively. The genotoxic mechanisms of action are of great biological significance, as for instance some oxidized and alkylated bases are potentially mutagenic (Grollman and Moriya, 1993; Shrivastav *et al*., 2009) and since that cross-linking agents are typically clastogenic (reviewed in Noll *et al*., 2006). Indeed, nowadays some of these mechanisms of action already have an impact in regulatory decision-making. Directly DNA damaging compounds are considered non-threshold carcinogens, and thus risk exist at any level of exposure, whereas indirect DNA damage through oxidative stress have threshold effect related with dose, and thus health-based guidance values can be established (EFSA, 2005).

Regarding AOPs, in the AOP-Wiki, which is supported by the OECD, some of these DNA lesions are included within its framework as MIEs or KEs (*e.g*., DNA alkylation or oxidative DNA damage) which are linked to AO such as heritable mutations in offspring or chromosomal aberrations (AOPWiki, 2020). As the number of AOPs is constantly increasing, it is of great importance to develop reliable tools and methods that allow the detection and measurement of the KEs. In this context, the comet assay modifications evaluated in this study are promising tools for in vitro genotoxicity assessment focused on the detection of different mechanisms of action, as we were able to detect and differentiate with high sensitivity oxidizing, alkylating and cross-linking agents.

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Chapter 7

General discussion

Genotoxicity evaluation is of key importance in the risk assessment process of substances to which humans may be exposed (*e.g.*, pharmaceutical drugs, food and feed additives, contaminants or pesticides, among others). Indeed, it has been long established that genotoxic compounds are able to damage DNA entailing severe consequences for human health such as mutations, which are strongly linked with carcinogenic processes (reviewed in Basu, 2018).

In this context, a mechanistic approach more relevant to humans is increasingly warranted for genotoxicological risk assessment. Indeed, new tools such as AOPs are expanding the number of potential endpoints, in which not only classical ones (*i.e.,* point mutations or chromosome aberrations) but also other mechanistic events, traditionally not considered as endpoints (*e.g.,* DNA oxidation), are integrated and contemplated as relevant factors in decision-making processes for hazard evaluation (Leist *et al*., 2017). In AOPs it is essential to identify the KEs involved in the pathway to the AO as well as to describe how that events can be measured. In this regard, having reliable tools and methods for its measurement is of great relevance. In relation to genotoxicity, the comet assay might be a promising method as it is able to detect premutagenic lesions that occur before the classical endpoints, such as oxidized bases or DNA cross-links, adding mechanistic information.

The standard alkaline comet assay (*i.e*., the version that detects SBs and ALS) on its *in vivo* version has an OECD guideline (OECD, 2016) and it is considered for the *in vivo* follow-up of positive genotoxic findings *in vitro* within EFSA and ICH genotoxicity testing strategies (EFSA, 2011; ICH, 2011). One of the main advantages of this technique is that it can be applied in most of the organs. Unlike the *in vivo* version, the *in vitro* comet assay has no OECD guideline and compared to the long-established *in vitro* genotoxicity assays considered within the genotoxicity testing strategies, it could be seen as not having additional value in risk assessment.

However, the main potential of the *in vitro* comet assay lies in the modification of its protocol to detect other DNA lesions, such as altered bases or cross-links. Indeed, considering the modified *in vitro* comet assay within regulatory processes is in line with the strategic inclusion of so-called New Approach Methodologies (NAMs) in human risk assessment (Parish *et al*., 2020). The term NAMs has a broad definition as it applies to alternative methods and approaches to animal testing that can be used to provide information in the context of risk assessment (*e.g., in silico* modelling or *in vitro* measurements). Ideally, information provided by NAMs should relate to a mechanistic basis or to understand the mechanism of action of a certain compound (*e.g.,* include evidence from the MIE or KE defined within an AOP) and thus NAMs may be integrated through the AOP approach (ECHA, 2016).

The use of lesion-specific endonucleases in combination with the comet assay is the most popular modification to the assay protocol. Particularly, the area in which the enzyme-modified comet assay has been used more extensively is, by far, *in vitro* genotoxicity testing, followed by human biomonitoring, *in vivo* genotoxicity testing and ecological studies (Muruzabal *et al*., 2021a; Chapter 3). Particularly, it is becoming more and more popular for the genotoxicity evaluation of NMs (reviewed in Collins *et al*., 2017). Moreover, the *in vivo* enzyme-modified comet assay is in fact considered useful by some agencies as a follow-up test for positive *in vitro* results or as a supplementary test for mechanistic evaluation (EFSA, 2011). Furthermore, despite not having an OECD guideline yet, EFSA recommends the use of the *in vitro* enzyme-modified comet assay to provide complementary information of the genotoxic mechanisms of action of NMs, especially for the detection of oxidative DNA lesions, as many NMs have shown to induce oxidative stress (EFSA, 2018).

Up to now, twelve different enzymes, from bacterial and human origin, have been used in combination with the comet assay for the detection of several DNA lesions, such as oxidized and alkylated bases, AP-sites, cyclobutane pyrimidine dimers or uracil misincorporation (Muruzabal *et al*., 2021a; Chapter 3). Among the twelve enzymes, the most frequently used are Fpg and Endo III, detecting oxidized purines and pyrimidines respectively. Additionally, it has been demonstrated that Fpg, apart from detecting oxidized bases, also detects ring-opened purines derived from some alkylated lesions at alkaline conditions (*e.g.*, during lysis at pH 10 in the comet assay) (Speit *et al*., 2004; Hansen *et al*., 2018). Indeed, Azqueta and colleagues showed in 2013 that Fpg enhanced the sensitivity of the comet assay without compromising the selectivity of the comet assay (Azqueta *et al*., 2013).

The comet assay has also been modified with enzymes for the detection of alkylated bases; AlkA was the first one (Collins *et al*., 2001) and a few years later AlkD was also applied (Hašplová *et al*., 2012). However, these enzymes are not commercially available and so most researchers do not have access to them or the facilities and knowledge for their production. Indeed, these enzymes have not been extensively used and there are no recent publications showing its use. In total, there are 16 publications using AlkA (most of them from the same group) and only 2 publications using AlkD, being the most recent ones published in 2006 (Dušinská *et al*., 2006) and 2013 (Ramos *et al*., 2013), respectively (Muruzabal *et al*., 2021a; Chapter 3). Recently, hAAG, a commercially available enzyme, was combined with the comet assay to detect alkylated bases (Muruzabal *et al*., 2020; Chapter 5). The enzyme was successfully applied and it was possible to detect different alkylated damage, from the very low control levels up to the saturation level of the comet assay.

Moreover, the Fpg-modified and the hAAG-modified comet assay versions were also compared under two different lysis conditions (*i.e.*, pH 7 and pH 10) and different substrates (untreated, $KBrO_3$- and MMS-treated cells) (Muruzabal *et al.*, 2020; Chapter 5). Our results showed that Fpg only detected MMS-derived lesions at alkaline lysis conditions, which induced the conversion of some alkylated bases (*e.g.*, N7-methylguanines) into ring-opened purines; whereas hAAG-sensitive sites were the same independently of the pH of the lysis solution. Considering all data, it seems that hAAG is detecting N7-methylguanines, the methylated ring-opened purines derived at high pH and the 3-methyladenines, which are also induced by MMS and are not detected by the Fpg-modified comet assay. Clearly, hAAG did not detect 8-oxoguanines induced by $KBrO_3$ at any conditions tested, and Fpg detected $KBrO_3$-derived lesions independently of the lysis pH.

It is known that hAAG is able to detect non-alkylated lesions, such as deaminated purine lesions (*i.e.*, hypoxanthine) and the lipid peroxidation-derived adduct 1,N6-ethenoadenine (Lee *et al.*, 2009; Taylor *et al.*, 2018). For this reason, we evaluated the incision capability of hAAG towards these and other oxidized DNA lesions using the Glyco-SPOT assay (multiplex oligonucleotide-cleavage assay), which confirmed its cleavage activity toward hypoxanthine and ethenoadenine (Muruzabal *et al.*, 2020; Chapter 5). Thus, this should be taken into account when interpreting the results as these lesions may also be detected by the hAAG-modified comet assay.

Moreover, it is worth mentioning that N-methylated bases (*e.g.,* N7-methylguanine) detected by hAAG are the most common form of alkylated lesions (Shrivastav *et al.*, 2009) but are less mutagenic compared to O-alkylations (Kondo *et al.*, 2010), which are generated in a much lesser extent and are of great biological significance due to its high mutagenic potential (Shrivastav *et al.*, 2009; Fu *et al.*, 2012). The lesion O6-methylguanine is a primary mutagenic lesion under most conditions and the enzyme responsible of its repair is O6-alkylguanine DNA alkylstransferase (also known as MGMT). Unfortunately, this enzyme cannot be combined with the comet assay to detect O-alkylations, as MGMT is a transferase, not a glycosylase, and the methyl group is transferred to an acceptor (Kaina *et al.*, 2007). Thus, in the reparation process the base is demethylated rather than removed, so the enzyme does not leave an AP-site or a SB that can be measured with the comet assay.

An important aspect to facilitate the implementation of the *in vitro* comet assay within the current risk assessment strategies is to ensure its reproducibility. Despite the wide use of the enzyme-modified comet assay in the scientific literature (Muruzabal *et al.*, 2021a; Chapter 3), an important factor that might hinder its application in a regulatory context, is the relatively high

inter-laboratory variation reported. Differences in the methods used by different research groups make hard to compare results obtained with the alkaline standard and the Fpg-modified comet assay in different laboratories (Forchhammer *et al*., 2012; Ersson *et al*., 2013). In addition, inter-experimental and intra-assay variation has also been reported in both versions of the assay (Møller *et al*., 2010). It should be noted that all the factors that influence the outcome of the standard assay, also influence the outcome of the enzyme-modified comet assay but in this case with an extra factor (the enzyme incubation) that may increase the inter-laboratory variation (Azqueta *et al*., 2019).

According to our results, apart from the enzyme concentration and time of incubation, that must be determined in titration experiments, special care should be taken when employing different throughput formats of the comet assay (*i.e.*, 2 gels/slide, 12 minigels/slide or 96 gels/Gelbond® film) as the way the enzyme incubation is performed is also critical (Muruzabal *et al*., 2019; Chapter 4). Indeed, the comparison of the response of Fpg employing 2 gels/slide and 12 minigels/slide formats showed that 10x lower enzyme concentrations are needed in the case of the 12 minigels/slide format compared with 2 gels/slide, as incubation approach and ratio of enzyme volume/volume of gel is different for each format. This can be explained as in the 2 gels/slide format, gels are incubated with the enzymes by adding a 50 µl drop on top of each gel (covered by a coverslip), whereas in the 12 minigels/slide format, each gel is isolated from each other in a different well (using the 12-Gel Comet Assay Unit™) and 30 µl of enzymes is added covering the gel. Thus, a higher ratio of enzyme volume per volume of gel is found in the 12 minigels/slide format. In addition, longer times of incubation (*e.g.,* from 30 min to 1 h) have a slight effect in the 2 gels/slide format but remarkable effect in the 12 minigels/slide format. In both cases, the enzyme is added keeping the slides ice-cold to prevent the activation of the enzyme before the slides are transferred to 37 °C. However, in the case of 12 minigels/slide a cooling effect is observed, as the base of the 12-Gel Comet Assay Unit™ is a bulky structure made of metal, so it needs more time to reach the proper temperature for the enzyme reaction. Therefore, titrations must be performed to assess enzyme concentration and time of incubation using the same format, protocol and equipment that is going to be used in forward experiments (Muruzabal *et al*., 2019; Chapter 4).

Cross-links cannot be detected using the standard comet assay and its detection using enzymes entails great difficulty, as although interstrand cross-links can be converted to DSBs during its repair, considerable uncertainty remains regarding the precise mechanisms (Chesner *et al*., 2017). Nevertheless, there is an easier approach to modify the comet assay for cross-links detection. This modification is based on the fact that cross-links, in the comet assay context,

have the opposite effect to SBs, as they inhibit the DNA migration during the electrophoresis. Therefore, the objective when using this modification is to measure the reduction in DNA migration. For this purpose, an additional treatment to induce SBs is performed in all samples to establish a known level of DNA damage (*e.g.,* 40-50% of DNA in tail). Thus, control cells will present the expected amount of DNA damage, whereas if the studied compound induces cross-links, treated cells will show lower amounts of DNA damage than the controls, as cross-links inhibit DNA migration.

Although less extended compared with the combination with enzymes, the use of the comet assay modification to detect cross-links has also been applied (Wu and Jones, 2012; Swift *et al*., 2020). However, according to our knowledge, neither the cross-links modification nor the enzyme-modified comet assay have been validated. To evaluate the potential of these modifications in detecting different DNA lesions, an internal validation was performed using different compounds with several mechanisms of action: oxidizing and alkylating agents, cross-linkers, a bulky-adducts inducer and non-genotoxic compounds. Particularly, four commercial enzymes, Fpg, hAAG, hOGG1 and Endo III, and a non-commercial version of Fpg were employed in the study, together with the modification to detect cross-link.

As mentioned before, Fpg is the most used enzyme in combination with the comet assay (Muruzabal *et al*., 2021a; Chapter 3). Both, a commercial and a non-commercial bacterial extract (from an overproducing *E. coli* strain), have been widely used in the comet assay. For that reason, both versions were used and compared in the validation study. Commercial hAAG was selected for the detection of alkylated bases, although as it was previously shown, it can also detect ethenoadenines and hypoxanthine (Muruzabal *et al*., 2020; Chapter 5). In addition, the commercial hOGG1 was employed to detect oxidized purines, mainly 8-oxoguanines, as it seems to be more specific to detect this lesion compared to Fpg (Smith *et al*., 2006). Finally, commercial Endo III was employed for the detection of oxidized pyrimidines. Interestingly, all enzymes were diluted with the same reaction buffer, which is very convenient for using all of them in the same experiment.

Recently, we have shown that the use of the enzyme-modified comet assay together with the cross-links modification increases significantly the comet assay ability to detect different premutagenic lesions, providing genotoxic mechanistic information about the type of damage, which potentially may gain further relevance in a regulatory context (Muruzabal *et al.,* 2021b; Chapter 6). It was possible to classify the genotoxic compounds according to their mechanisms of action, as they were detected by the expected modification. Non-genotoxic compounds,

including the cytotoxic one, did not induce false positives in any of the modifications tested. As expected, it was not possible to detect bulky DNA adducts with any of the modifications employed. An attempt to detect this lesion using enzymes has already been performed (Dušinská and Collins, 1996), although it did not give satisfactory results. However, it has been described other comet assay modification to detect bulky and DNA distorting adducts by using DNA repair inhibitors (Gedik *et al*., 1992; Vande Loock *et al*., 2010; Ngo *et al*., 2020).

Detecting these genotoxic mechanisms of action is of great biologic significance (Grollman and Moriya, 1993; Noll *et al*., 2006; Shrivastav *et al*., 2009) and has already an impact in regulatory decisions. Direct DNA damaging compounds such as cross-linkers and alkylated agents, are considered non-threshold compounds, and thus some risk may exist at any level of exposure, whereas indirect DNA damage through oxidative stress have threshold effect related with dose, and thus health-based guidance values can be established.

It is worth to mention that many of these lesions can be detected by employing different analytical techniques (*e.g.,* mass spectrometry or high-performance liquid chromatography) or biochemical methods (*e.g.,* immunoassays or immunohistochemistry techniques). Considering analytical techniques, specialized (and highly expensive) equipment along with internal standards are required. On the other, hand a risk of cross-reactivity with other DNA adducts and poor identification is found when employing antibody-based methods (reviewed in Himmelstein *et al*., 2009). Moreover, some studies led by the European Standards Committee on Oxidative DNA Damage (ESCODD) compared the performance of the Fpg-modified comet assay with chromatographic methods (*e.g.,* high performance liquid chromatography -HPLC-) in detecting 8-oxoguanines in samples from different sources (*e.g.,* liver tissue or culture cells) (ESCODD, 2002a, b, 2003; ESCODD *et al*., 2005). Results showed that despite HPLC was very precise and good at measuring high levels of 8-oxoguanines, when analyzing low levels serious and variable artefact of 8-oxoGua were introduced into the DNA during sample preparation. On the other hand, the Fpg-modified comet assay although less precise, was more accurate at measuring low and background levels of 8-oxoguanines (ESCODD, 2002a, b, 2003; ESCODD *et al*., 2005).

In Chapter 6, the enzyme-modified comet assay using 5 different enzymes and the cross-links modification were performed in two different experiments: one involving the use of all enzymes, in which we also detected SBs and ALS, and the other one using the modification to detect cross-links. However, since the experiments were performed using the medium throughput version of the assay which uses 12 minigels/slide, the integration of all the modifications in a single experiment is completely feasible. Indeed, a protocol including both modifications within a

single-integrated experiment has been already prepared and successfully applied. Moreover, using this throughput format it would be possible to include more enzymes in order to cover a higher spectrum of DNA lesions, such as T4 Endo V (for cyclobutane pyrimidine dimers) or Udg (to detect uracil residue) (Muruzabal *et al*., 2021; Chapter 3); and it would also be possible to encompass the modification to detect bulky DNA adducts. The possibility to detect all these lesions in a single experiment and using the same material and equipment gives this approach a huge advantage over other techniques.

The modifications studied in this thesis can be easily applied in *in vivo* genotoxicity testing or even in human and ecological biomonitoring. For instance, a recent article reviews publications in which enzymes have been employed *in vivo*, and makes the case for an extension of the existing OECD guideline to include the enzyme modification (Collins *et al*., 2020). Moreover, the modification for cross-links detection has been successfully applied *in vivo* (Pant *et* al., 2015; Richterova *et al*., 2018). Nevertheless, in all these cases additional validation would be required using several compounds with various mechanisms of action, as in most cases only single compounds were analyzed.

We consider that this thesis establish the basis of a promising future in which the *in vitro* comet assay along with its modifications may gain relevance in a regulatory context in which a more human-relevant mechanistic toxicology is being demanded. However, much work is still required, as a complete validation with more compounds with several and different mechanisms of action as well as an interlaboratory evaluation of the modifications need to be done. Indeed, the complete validation is essential to facilitate the way through an OECD TG of the *in vitro* comet assay, which would be extremely useful for its further implementation to detect relevant genotoxic mechanisms of action within hazard identification and risk assessment strategies.

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Chapter 8

Conclusions

1. A review of all enzymes that have been employed in combination with the comet assay was prepared and it was concluded that:

    1.1. Twelve enzymes that detect oxidized purines and pyrimidines, alkylated bases, ring-opened purines, cyclobutane pyrimidine dimers, uracil residue, AP-sites, hypoxanthine and ethenoadenines have been used in combination with the comet assay. Fpg, used for detecting oxidized purines, is, by far, the most commonly used enzyme, followed by Endo III and hOGG1, which detect oxidized pyrimidines and purines respectively.

    1.2. The area of application in which the enzyme-modified comet assay has been more widely used is in *in vitro* genotoxicity testing to assess oxidative DNA damage. Its use is also extended in human biomonitoring studies and, to a lesser extent, in *in vivo* genotoxicity testing. In ecological studies, such as ecotoxicology and environmental biomonitoring, its use is marginal.

2. Regarding the effect of the enzyme incubation conditions in the outcome of the Fpg-modified comet assay when using different throughput formats:

    2.1. Different enzyme concentrations and times of incubation, carefully selected in titration experiments, are required in the 2 gels/slide format and the 12 minigels/slide.

    2.2. Titration experiments should be carried out using the same format, protocol and equipment that is going to be used in the following experiments.

3. Regarding the use of the commercially available hAAG in the comet assay:

    3.1. hAAG appears to detect 7-methylguanines and its derived ring-opened purines, 3-methyladenine and some ethylated bases induced by MMS and EMS when combined with the alkaline comet assay. It does not detect oxidized bases.

    3.2. hAAG detects hypoxanthine and ethenoadenines in an oligonucleotide cleavage assay, which should be considered when interpreting the results.

4. Regarding the internal validation of the enzyme-modified comet assay using different enzymes and the modification of the comet assay for cross-links detection:

    4.1. The enzyme-modified comet assay using Fpg, hAAG, hOGG1 and Endo III is able to specifically differentiate some oxidized and alkylated DNA lesions allowing the classification of compounds with different mechanisms of action.

    4.2. A commercial version of Fpg and a non-commercial Fpg obtained from an over-producing *E. coli* strain, if properly titrated, show the same performance.

    4.3. The modification of the comet assay for cross-links detection is able to specifically detect cross-linking agents.

    4.4. The comet assay in combination with Fpg, hAAG, hOGG1 and Endo III together with the cross-links modification can be performed on a single assay.

5. The modified *in vitro* comet assay, under a unique and standardized protocol, is suggested as a promising tool for *in vitro* mechanistic genotoxicity assessment able to detect and differentiate oxidizing, alkylating and cross-linking agents. However, further validation steps need to be carried out in the future.

Dissemination of the results

1. Publications

- *The enzyme-modified comet assay: Enzyme incubation step in 2 vs 12-gels/slide systems*. Muruzabal, D., Langie, S., Pourrut, B., Azqueta, A. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* (2019), 845: 402981. doi: 10.1016/j.mrgentox.2018.11.005
- *Novel approach for the detection of alkylated bases using the enzyme-modified comet assay*. Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Gützkow, KB., López de Cerain, A., Vettorazzi, A., Azqueta, A. *Toxicology Letters* (2020), 330: 108-117. doi: 10.1016/j.toxlet.2020.04.021
- *The enzyme-modified comet assay: Past, present and future*. Muruzabal, D., Collins, A., Azqueta, A. *Food and Chemical Toxicology* (2021), 147: 111865. doi: 10.1016/j.fct.2020.111865
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2. Conferences

- *Testing commercial enzymes in combination with the Comet assay* (Poster). Muruzabal, D., López de Cerain, A., Vettorazzi, A., Azqueta, A. *XI Jornada de la investigación en ciencias experimentales y de la salud de la Universidad de Navarra*, 23rd of March 2018, Pamplona (Navarra, Spain).
- *The enzyme-modified comet assay: Measuring oxidized and alkylated bases using the 12-gels format* (Oral communication). Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., López de Cerain, A., Vettorazzi, A., Azqueta, A. *XXIV Scientific meeting of the Spanish society of environmental mutagenesis (SEMA)*, 20th-22nd of June 2018, Madrid (Spain).
- *The Fpg-modified comet assay: protocols in use, critical points and recommendations* (Oral communication). Azqueta, A., Muruzabal, D., Boutet-Robinet, E., Møller, P., Collins, A. *4th International Congress on Occupational & Environmental Toxicology (ICOETox)*, 24th-26th of October 2018, Matosinhos (Porto, Portugal).
- *Is it possible to detect alkylated DNA damage using the enzyme-modified comet assay?* (Poster). Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., López de Cerain, A., Vettorazzi, A., Azqueta, A. *XII Jornada de la investigación en ciencias experimentales y de la salud de la Universidad de Navarra*, 11th of April 2019, Pamplona (Navarra, Spain).
- *Novel approach for the detection of alkylated bases using the enzyme-modified comet assay* (Oral communication). Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., López de

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- *High-throughput comet assay* (Oral communication). Azqueta, A., Muruzabal, D., Brunborg, G., Gützkow, KB., Shaposhnikov, S., Collins, A. 47th *European Environmental Mutagenesis and Genomics Society (EEMGS) Meeting*, 19th-23rd of May 2019, Rennes (France).
- *Detección de bases alquiladas mediante el ensayo del cometa* (Oral communication). Muruzabal, D., Sanz-Serrano, J., Sauvaigo, S., Treillard, B., López de Cerain, A., Vettorazzi, A., Azqueta, A. *IV Workshop de la red de excelencia MICOFOOD*, 29th-31st of May 2019, Pamplona (Navarra, Spain).
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