Universidad de Navarra

Facultad de Farmacia y Nutrición

Comet assay modifications for its application in food safety

Modificaciones del ensayo del cometa para su aplicación en seguridad alimentaria

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El presente trabajo de investigación titulado:

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que presenta D. José Manuel Enciso Gadea para aspirar al grado de Doctor por la Universidad de Navarra en el Programa de Doctorado en Alimentación, Fisiología y Salud, ha sido realizado en el Departamento de Farmacología y Toxicología de la Facultad de Farmacia y Nutrición, bajo la dirección de la Dra. Adela López de Cerain Salsamendi y la co-dirección de la Dra. Amaya Azqueta Oscoz y la Dra. Ariane Vettorazzi Armental.

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Summary

Information on genotoxicity is of a key importance for the toxicological characterisation of different compounds. In this vein, and due to its various advantages, the comet assay is currently included in the genotoxicity testing strategy used in the food safety area. However, improvement points of particular interest have been identified. Thereby, the main objective of the present work was to evaluate some critical points of the comet assay, such as the time of lysis, *in vitro*, and the methodology used in the freezing/thawing procedures of tissue samples, their stability and the application of the Fpg-modified assay, *in vivo*. In addition, the *in vivo* comet assay was applied to frozen kidney samples obtained in a previous repeated-dose toxicity study of the food contaminant ochratoxin A. Finally, the knowledge derived from these objectives resulted in the development of standard operating procedures for both the *in vitro* and *in vivo* comet assays, which could be applied in good laboratory practice studies.

Keywords: comet assay, genotoxicity, food safety, ochratoxin A

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List of abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxoGua	8-oxo-7,8-dihydroguanine
8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AAF	2-acetylaminofluorene
Alk A	3-methyladenine glycosylase
ALS	alkali-labile sites
AP	apurinic/apyrimidinic
COLIPA	European Cosmetics Industry Association
COM	Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
CPN	cisplatin
CV	coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethylsulfoxide
EC	Enzyme Commission
ECVAG	European Comet Assay Validation Group
ECVAM	European Centre for the Validation of Alternative Methods
EFPIA	European Federation of Pharmaceutical Industries and Associations
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EMS	ethyl methanesulfonate
Endo III	Endonuclease III
ESCODD	European Standards Committee on Oxidative DNA Damage
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
F344	Fischer 344
FDA	Food and Drug Administration
FDA/CDER	U.S. Food and Drug Administration Centre for Drug Evaluation and Research
Fe/NTA	ferric nitriloacetate
Fpg	Formamidopyrimidine-DNA glycosylase
GLP	Good Laboratory Practice
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase

IARC	Internacional Agency for Research on Cancer
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ITS	Integrated Testing Strategy
IWGT	International Comet Assay Workshop on Genotoxicity Testing
JCR	Journal Citation Reports
JaCVAM	Japanese Center for the Validation of Alternative Methods
JEMS/MMS	Japanese Environmental Mutagen Society/Mammalian Mutagenesis Study Group
MMS	methyl methanesulfonate
MN	micronucleus
MTD	maximum tolerated dose
NICEATM	U.S. NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OEEC	Organisation for European Economic Cooperation
OGG1	8-oxoguanine DNA glycosylase
OTA	ochratoxin A
OTHQ	ochratoxin A hydroquinone
OTHQ-GSH	ochratoxin A hydroquinone glutathione conjugate
OTQ	ochratoxin A quinone
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical Substances
ROS	reactive oxygen species
RSMN	reconstructed skin micronucleus
SD	standard deviation
SOD	superoxide dismutase
SBs	strand breaks
TCVG	*S*-(1,2,2-trichlorovinyl) glutathione
TG	Test Guideline
tGSH	total glutathione
UDG	uracil DNA glycosylase
UDS	unscheduled DNA synthesis
UV	ultraviolet

VSMC	vascular smooth muscle cells
WHO	World Health Organization

Chapter 1

General introduction

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1. DNA damage and mutagenicity

DNA damage is defined as an alteration in the chemical structure of DNA, which occurs spontaneously, either as a result of the natural ageing process of cells (*i.e.*, by endogenous agents), or as a consequence of the interaction with exogenous agents (*i.e.*, xenobiotics). Therefore, DNA is not as stable as we tend to think, as a wide variety of both endogenous and exogenous agents can induce various kinds of DNA damage, such as single and double strand breaks (SSBs and DSBs, respectively), oxidised and alkylated bases, bulky adducts, intra- and inter-strand cross-links, adducts on the phosphodiester backbone and protein-DNA cross-links (see Figure 1).

When a xenobiotic compound has the ability to damage DNA, it is considered genotoxic. Thereby, the term genotoxicity refers to the property of chemical agents to damage genetic information (DNA) and/or other cellular components which regulate the fidelity of the genome.

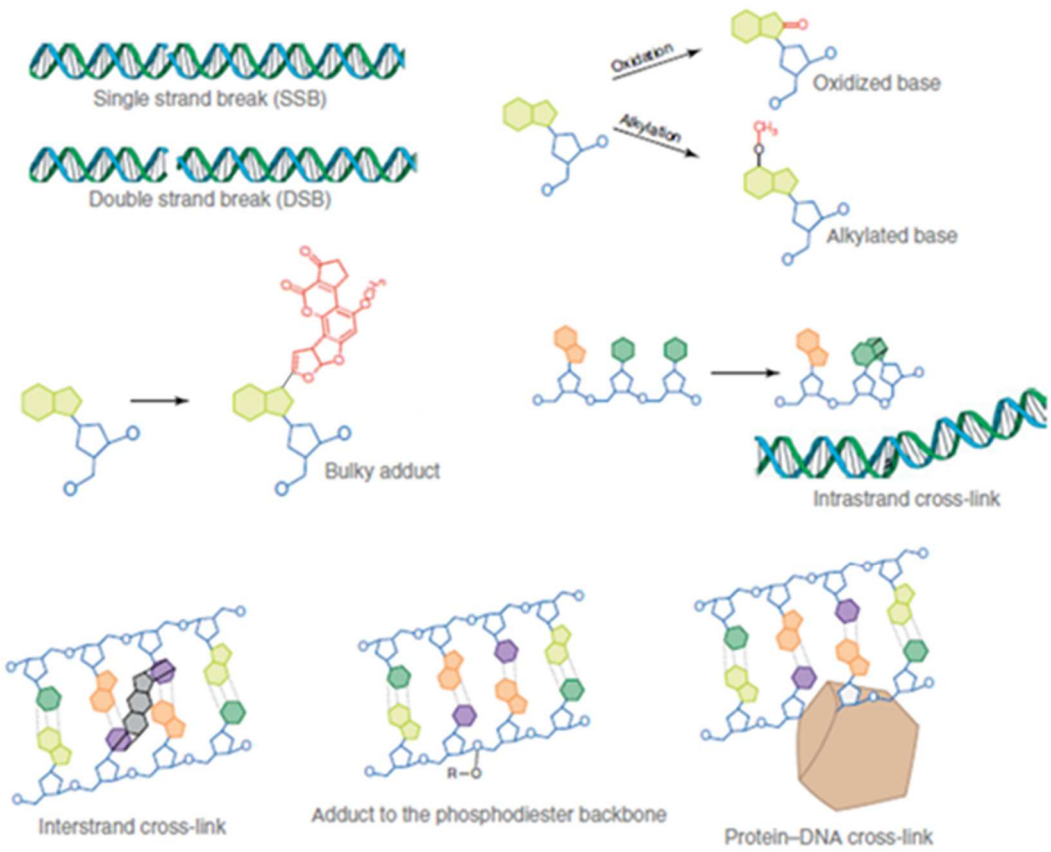

Figure 1. Schematic diagrams of different DNA lesions. Adapted from Azqueta and Collins, 2011.

DNA damage can have disruptive effects on transcription, DNA replication and chromosome segregation. A high level of DNA damage tends to trigger apoptosis, while lower levels are dealt with by effective DNA repair pathways. However, some DNA damage may remain unrepaired (or be misrepaired) when the cell replicates DNA, providing the basis for mutations, that are defined as stable changes in DNA sequence that can be transmitted to the offspring.

As one may notice, genotoxicity and mutagenicity are pretty closed terms. As defined in the European Food Safety Authority (EFSA) Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011), "mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms". Traditionally, these changes are classified as gene mutations, which involve a single gene, or as chromosome mutations, involving a block of genes or chromosomes. More specifically, gene mutations are caused by a single base pair substitution or a deletion or insertion of a few base pairs (frameshift mutations). On the other hand, chromosome mutations can be divided into structural chromosome aberrations, produced by agents capable of causing breaks in chromosomes that result in the loss or rearrangements of chromosome segments (*i.e.*, a clastogens), and numerical chromosome aberrations, produced by agents giving rise to a change (gain or loss) in chromosome number in cells (*i.e.*, an aneugen) (see Figure 2).

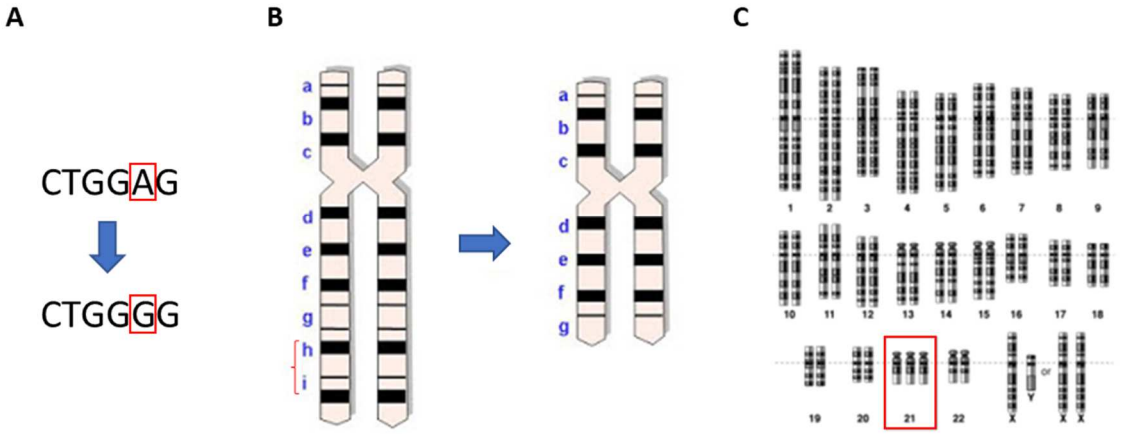

Figure 2. Examples of gene mutation (A), structural chromosome (B) and numerical chromosome (C) aberrations.

Thus, fixation of DNA damage results in mutagenic effects that are generally considered to be essential for heritable effects and in the multi-step process of malignancy (ICH, 2012). Genetic alterations (*i.e.*, both germ-line and somatic mutations, such as base pair changes, insertions/deletions, short tandem repeat expansions, copy number variants, transposon-

mediated mutations and chromosome mutations) have been found to be important in human disease. Mutations in germ cells can lead to spontaneous abortions, infertility or heritable damage to the offspring and possibly to the subsequent generations. For example, three "number 21" chromosomes or trisomy 21, a form of aneuploidy, is characteristic of the Down syndrome.

Cancer is a disease of somatic cells which is strongly linked to the occurrence of mutations. Somatic mutations may cause cancer if they occur in proto-oncogenes, tumour suppressor genes and/or DNA damage response genes. In fact, their role in the causation of cancer has been reviewed on several occasions (Lengauer *et al.*, 1998) and has been proven, among others, for retinoblastoma (Knudson, 1971), neuroblastoma and pheochromocytoma (Knudson and Strong, 1972), and colorectal cancer (Ma *et al.*, 2017).

While the contribution of somatic mutations in the development of a cancer is considered essential and has been thoroughly studied, their role in non-malignant diseases (*e.g.*, neurofibromatosis 1 and 2, McCune-Albright disease, paroxysmal nocturnal hemoglobinuria, incontinentia pigmenti in males, and many others affecting the central nervous system, heart and kidney) has only been confirmed recently due to the advances in molecular genetics (Erickson, 2010, 2003).

In summary, all mutagenic compounds are genotoxic, as it is assumed that a mutation is present because a DNA lesion occurred previously. Thus, DNA lesions are also called pre-mutagenic lesions. However, it is important to note that DNA damage reflects a dynamic steady state, in which the input of damage is normally balanced by the output (*i.e.*, DNA repair).

2. European Food Safety Authority strategy for genotoxicity testing

Information on genotoxicity is essential not only for the risk assessment of pharmaceutical drugs, agrochemicals or industrial chemicals, but also of natural and environmental contaminants occurring in food and feed. Many regulatory agencies and advisory bodies have made recommendations on genotoxicity testing strategies. In the field of pharmaceutical drugs, the International Council for Harmonisation (formerly the International Conference on Harmonisation) of Technical Requirements for Pharmaceuticals for Human Use (ICH) has developed guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (ICH, 2012). This document was approved in its final version in 2011, resulting from the fusion of two previous documents that were approved in 1995 and 1997.

More recently, in the field of food and feed, EFSA has provided a Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011) and a Guidance for submission for food additive evaluations (EFSA, 2012).

EFSA is a European agency which was funded by the European Union under the General Food Law (Regulation 178/2002). It was set up in 2002 following a series of food crises in the late 1990s, with the aim of providing scientific advice and communication on risks associated with the food chain. Their competence in the risk assessment process covers the areas of food and feed safety, nutrition, animal health and welfare, plant protection and plant health (EFSA, 2017).

As stated in the latest version of the Guidance for submission for food additive evaluations (EFSA, 2012), published on August 2012, and elaborated by the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), genotoxicity testing for risk assessment of substances in food and feed is performed with the following aims:

- to identify substances which could cause heritable damage in humans,
- to predict potential genotoxic carcinogens in cases where carcinogenicity data are not available, and
- to contribute to understanding of the mechanism of action of chemical carcinogens.

The document encompasses the description of the data requirements for authorisation of a new food additive or a modification of an already authorised one, and a description of the risk assessment paradigm applied, which includes hazard identification, hazard characterisation, exposure assessment and risk characterisation. It is divided into five sections: chemistry and specifications, information on existing authorisations and evaluations, proposed uses and exposure assessment, toxicological studies and supplementary information. Focusing on the toxicological studies (toxicokinetics and toxicity) section, it is divided into the following five subjects: toxicokinetics (ADME), genotoxicity, toxicity testing (subchronic, chronic and carcinogenicity), reproductive and developmental toxicology and additional studies. General considerations are given, and a tier approach is proposed for each one of these aspects. Finally, a combined tiered approach consisting in 3 tiers (see Figure 3) was designed to evaluate toxicokinetics, genotoxicity, toxicity (including subchronic toxicity, chronic toxicity and carcinogenicity), and reproductive and developmental toxicity (*i.e.*, the core areas). Tier 1 was developed as a minimal dataset applicable to all compounds, while Tier 2 applies to compounds which are absorbed and/or demonstrate toxicity in a 90-day toxicity study or *in*

vitro genotoxicity in Tier 1 tests. Tier 3 testing should be performed on a case-by-case basis to elucidate specific endpoints needing further investigation of findings in Tier 2 tests.

This approach takes also into account the 3-Rs (replacement, refinement and reduction) animal testing strategy.

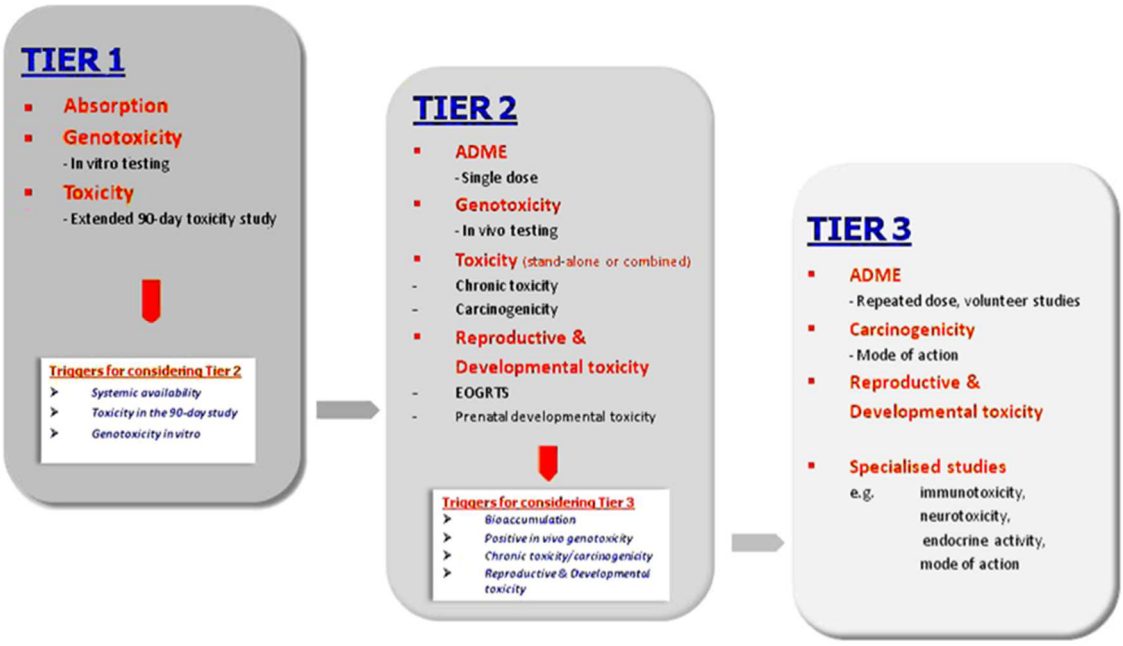

Figure 3. Tiered toxicity testing for food additives. From EFSA, 2012.

Testing for genotoxicity is considered a key aspect to move from Tier 1 to Tier 2, and also from Tier 2 to Tier 3. *In vitro* genotoxicity tests are applied in Tier 1 whereas *in vivo* genotoxicity tests are used in Tier 2.

As it is the main topic of the present work, special focus is given to the genotoxicity strategy proposed by EFSA , which is based on the Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011).

The Scientific Committee recommends a step-wise approach for the generation and evaluation of data on genotoxic potential (see Figure 4), which allows to assess the different endpoints implicated in carcinogenesis and heritable diseases. All the assays indicated in the figure, except the *in vivo* COMET assay, are identified by the corresponding Organisation for Economic Co-operation and Development (OECD) Test Guideline. The OECD Guideline for the *in vivo* COMET assay (OECD, 2016a) was approved later than the EFSA Scientific Opinion, and for this reason, it is not specified in the EFSA's figure. These documents will be explained in the next section.

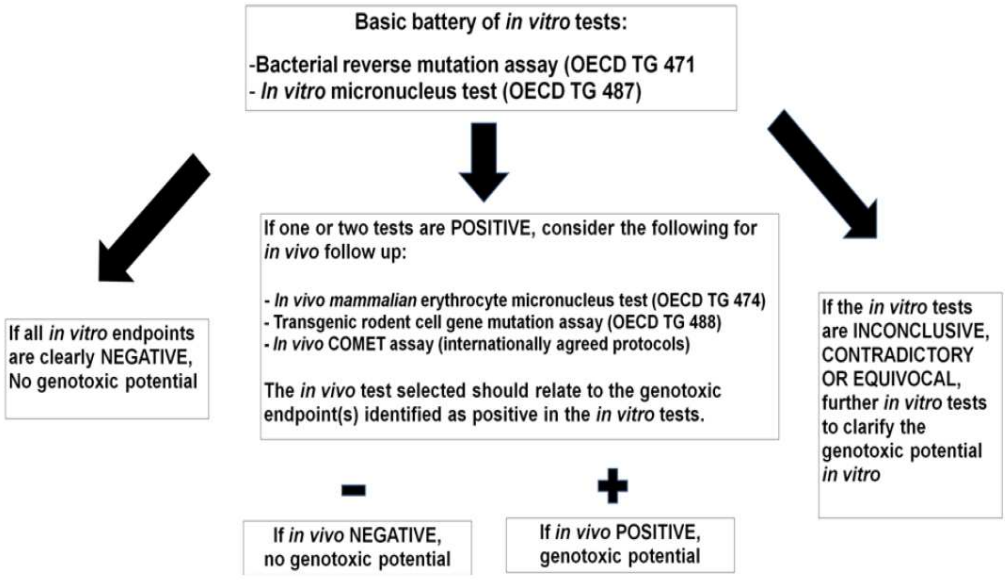

Figure 4. Schematic representation of the genotoxicity testing strategy recommended by the EFSA Scientific Committee. From EFSA, 2011. For references: OECD TG 471 (OECD, 1997); OECD TG 487 (OECD, 2016b); OECD TG 474 (OECD, 2016c); OECD TG 488 (OECD, 2013).

For initial screening of substances for genotoxic potential, the *in vitro* core test battery should be able to detect the three important genotoxic endpoints; that is to say: gene mutations, structural chromosome aberrations (*i.e.*, clastogenicity) and numerical chromosome aberrations (*i.e.*, aneuploidy). The Scientific opinion proposes the bacterial reverse mutation assay, which covers gene mutations, and the *in vitro* micronucleus test, which covers the endpoints of structural and numerical chromosome aberrations. Both *in vitro tests* should be conducted with and without an appropriate metabolic activation system, and cytotoxicity needs to be controlled. Further mammalian cells *in vitro* tests are not included, since it has been shown to reduce specificity with no substantial gain of sensitivity (EFSA, 2011).

After these two *in vitro* assays have been performed, there are three possibilities:

a) If all *in vitro* endpoints are clearly negative, then it can be concluded with reasonable certainty that the substance has no genotoxic potential. However, the Scientific Committee noted that, in rare exceptions, a small number of substances that are negative *in vitro* have positive results *in vivo*. Therefore, proceeding to *in vivo* testing with negative *in vitro* results should be considered case-by-case.
b) In the case of inconclusive, contradictory or equivocal *in vitro* results, further *in vitro* testing may clarify the genotoxic potential *in vitro*.

c) One or more positive *in vitro* tests require follow up by *in vivo* testing, which would allow to conclude on the genotoxic potential of the test substance. Several *in vivo* follow-up approaches are proposed in the document and are explained below. *In vivo* studies should be chosen in relation to the genotoxic endpoint(s) identified *in vitro*, as well as by knowledge of bioavailability, reactivity, metabolism and target organ specificity of the test substance. They should be performed in appropriate target organs or tissues, demonstrating that the agent reaches the tissue under investigation, and adopting a step-wise approach. If the first study is positive, no further test would be needed, and the test substance would be considered as an *in vivo* genotoxin; if it is negative, it can be concluded that the test substance is not an *in vivo* genotoxin. However, a second *in vivo* test on an alternative tissue might be necessary if it becomes apparent that the substance did not reach the target tissue in the first test. Also, an *in vivo* test on a second endpoint may be necessary if more than one *in vitro* test is positive.

The Scientific opinion considers the following *in vivo* tests for follow-up of *in vitro* positives:

- The *in vivo* mammalian erythrocyte micronucleus test (OECD, 2016c), or alternatively, the *in vivo* mammalian bone marrow chromosome aberration test (OECD, 2016d). Any of these assays is an appropriate follow-up for *in vitro* clastogens and aneugens.
- A transgenic rodent somatic and germ cell gene mutation assays (OECD, 2013), that can detect point mutations and small deletions, would be appropriate to follow-up *in vitro* gene mutagens.
- The *in vivo* comet assay (OECD, 2016a), considered a useful indicator test in terms of its sensitivity to substances which cause gene mutations and/or structural chromosome aberrations (*i.e.*, gene mutagens and clastogens, but not aneugens), has the advantage of being virtually applicable to any target tissue.

Both the transgenic rodent mutation assay and the *in vivo* comet assay would be suitable as a follow-up for *in vitro* gene mutation positives, and for detecting first site of contact effects. However, the Scientific opinion clarifies that, while the first one measures gene mutations directly, the comet assay is an indicator test for DNA lesions that may or may not result in mutations.

Finally, the Scientific Committee concluded that routine testing for genotoxicity in germ cells is not necessary, and it also recommends a documented weight-of-evidence approach to the

evaluation and interpretation of genotoxicity data, which takes into account other relevant data such as physico-chemical characteristics, structure-activity relationships, ADME, and the outcomes of any repeated-dose toxicity and carcinogenicity studies.

Historically, the genetic toxicology testing battery has been designed to be used as a surrogate for carcinogenicity testing. However, clear evidence of genotoxicity in somatic cells *in vivo* must be considered an adverse effect *per se*, even if the results of cancer bioassays are negative, since genotoxicity is also implicated in other somatic diseases than cancer (see section 1).

3. The OECD Guidelines for the Testing of Chemicals

The OECD is an intergovernmental economic organisation, established in 1948 as the Organisation for European Economic Cooperation (OEEC) to run the US-financed Marshall Plan for reconstruction after World War II. In general terms, the mission of the Organisation is to promote policies designed to improve the economic and social well-being of people around the world. Among many other things, the Organisation sets international standards on a wide range of areas, from agriculture and tax to the safety of chemicals (OECD, 2017a).

Each year, hundreds of new chemicals (*i.e.*, industrial chemicals, pesticides, food additives, biotechnology products and pharmaceuticals) reach the market. The OECD assists countries in harmonising test methods for chemical safety and good laboratory practice. For this purpose, since 1981, OECD member and partner countries have been developing the OECD Guidelines for the Testing of Chemicals, a collection of the most relevant internationally agreed testing methods used by governments, industry and independent laboratories to assess the safety of chemical products, with the aim to (OECD, 2017b):

- enhance the validity and international acceptance of test data;
- make the best use of available resources in both governments and industry;
- avoid the unnecessary use of laboratory animals;
- minimise non-tariff trade barriers.

In concrete terms, the OECD Test Guidelines (OECD, 2017b):

- Cover safety testing of chemicals in its broadest sense, including physical-chemical properties and effects on different systems.
- Are internationally accepted as standard methods for safety testing and provide the common basis for the Mutual Acceptance of Data, which implies the acceptance of the

data generated, in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP), in other OECD countries and partner countries adhered to the Decision of the Council, avoiding duplicative testing. GLP sets the quality standards for the organisation and management of test facilities and for performing and reporting studies related to the safety of chemical substances and preparations. Thus, they help to ensure that studies submitted to regulatory authorities, to notify or register chemicals, are of sufficient quality and rigour and are verifiable.

- Are essential for professionals working in industry, academia and government, and constitute a potent tool to be used not only in regulatory safety testing and in subsequent chemical product notification, registration and evaluation, but also in the selection and ranking of candidate chemicals during development, and in toxicology research, ensuring high-quality and reliable data.
- Aim to reflect the current state-of-the-art in hazard identification and characterisation testing. For this purpose, these Guidelines are regularly updated with the assistance of thousands of national experts from OECD member countries.

The OECD Guidelines for the Testing of Chemicals are divided into 5 sections: Physical-Chemical Properties, Effects on Biotic Systems, Environmental Fate and Behaviour (formerly called Degradation and Accumulation), Health Effects, and Other Test Guidelines. Besides, there is a complete set of the series on OECD Principles of GLP. Within Section 4, Health Effects, the guidelines for both *in vitro* and *in vivo* genotoxicity assays can be found (OECD, 2017c). Most of them have been recently revised. A summary of the genotoxicity assays included in the EFSA Scientific Opinion (EFSA, 2011) is presented in Table I.

Table I. *In vitro* and *in vivo* OECD Guidelines included in the EFSA Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011).

OECD Guideline	Title	First adopted	Last version adopted
In vitro			
Test No. 471	Bacterial Reverse Mutation Assay	1983	1997
Test No. 487	*In Vitro* Mammalian Cell Micronucleus Test	2010	2016
In vivo			
Test No. 474	Mammalian Erythrocyte Micronucleus Test	1983	2016
Test No. 475	Mammalian Bone Marrow Chromosomal Aberration Test	1984	2016
Test No. 488	Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays	2011	2013
Test No. 489	*In Vivo* Mammalian Alkaline Comet Assay	2014	2016

For references: Test No. 471 (OECD, 1997); Test No. 487 (OECD, 2016b); Test No. 474 (OECD, 2016c); Test No. 475 (OECD, 2016d); Test No. 488 (OECD, 2013); Test No. 489 (OECD, 2016a).

Basically, the structure of the OECD Guidelines is the same in all these cases. They contain a brief introduction, followed by initial considerations (and limitations) of the assay, a description of the verification of laboratory proficiency, the principle and description of the method sections, recommendations on the procedure, and finally, the data and reporting section. However, it should be noted that they provide minimum criteria for the acceptance of studies, and, therefore, additional requirements might be needed for each study (EFSA, 2012).

4. The comet assay

Östling and Johanson were the first to develop a microgel electrophoresis technique for detecting DNA damage at the level of the single cell in 1984 (Ostling and Johanson, 1984). However, it was not until 1988 that the most widely used protocol was described by increasing the alkalinity of the electrophoresis buffer to pH >13 (Singh *et al.*, 1988). This technique is nowadays known as the single-cell gel electrophoresis assay or, more commonly, the comet assay.

Under alkaline conditions (pH >13), the comet assay is able to detect single and double DNA strand breaks (SBs), as well as alkali-labile sites (ALS), notably the apurinic/apyrimidinic (AP) sites that are left when a base is lost from the DNA.

The scientific basis underlying the comet assay is relatively simple. Briefly, cells are immobilised in agarose on a support (a glass microscope slide or a plastic film) and lysed in a high salt solution that also contains detergent. This solution removes membranes, soluble cytoplasmic components and histones, to obtain nucleoids (*i.e.*, DNA attached at intervals to the nuclear matrix as series of loops) (Cook *et al.*, 1976). After an alkaline unwinding, DNA is denatured because of the disruption of hydrogen bonds between double-stranded DNA at pH values above 12.0 (Kohn, 1991). During this process, if strand breaks are present, the ultra-structure of DNA as supercoiled loops is partially relaxed, thus being able to migrate towards the anode during electrophoresis. After staining with a suitable dye and visualising under the fluorescence microscope, the relative amount of DNA which has been able to migrate is quantified (usually as % tail DNA) by either manual, semi-automated or fully-automated scoring methods, reflecting the frequency of DNA SBs in each cell. The term 'comet' is therefore used to identify the individual cell DNA-migration patterns produced by this assay, which resemble stellar comets when visualised under the fluorescence microscope (Figure 5). Usually, about 100 comets are evaluated per cellular sample.

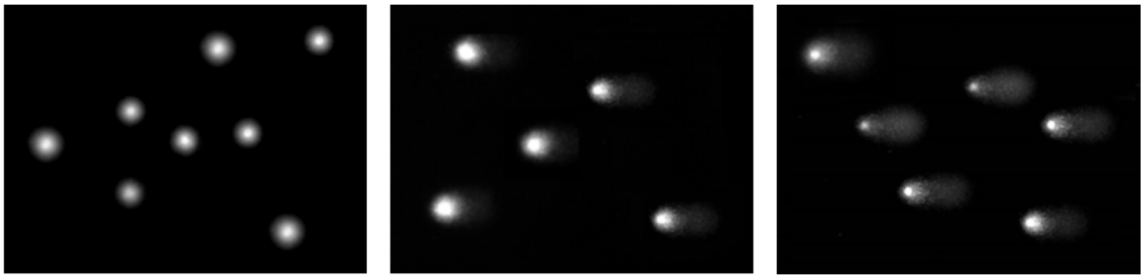

Figure 5. Comet images of TK6 cells with different levels of DNA damage, from no damage in the left to medium and high damage.

As mentioned before, the % tail DNA is the most commonly used parameter to describe a comet and so to describe a cellular sample by calculating the mean or the median of the % tail DNA of the evaluated comets. Another possibility is to express DNA damage in terms of actual DNA break frequency (*e.g.*, 'breaks per 10^6 base pairs' or 'breaks per cell'), which can be done by extrapolation of the % tail DNA from a calibration curve of cells exposed to ionising radiation (1 Gy of X- or ɣ-irradiation introduces 0.31 breaks per 109 Daltons of DNA; Ahnström and Erixon, 1981).

The comet assay has several advantages in comparison with other genotoxicity assays, mainly the possibility to apply it to any cell suspension, including non-dividing cells and tissues from which a single cell/nuclei suspension can be obtained. Moreover, results are obtained at the

level of the single cell, thus providing information about the heterogeneity in sensitivity or response between cells, and a small number of cells is needed. Besides, the comet assay is a very sensitive technique which detects low levels of DNA damage (*i.e.*, a few hundred DNA breaks per cell) (Tice *et al.*, 2000).

From a more practical point of view, the comet assay has also several advantages: it is a low-cost assay, feasible to be incorporated in many laboratories since very specific equipment (apart from a fluorescence microscope) is not required, and a relatively short-time period is needed to complete an experiment.

The comet assay has many different applications other than *in vitro* and *in vivo* genotoxicity testing. This technique is widely used in human biomonitoring and ecogenotoxicology and, during the last years, it has also been used in clinical applications at a research level. Moreover, it is also used in basic research into mechanisms of DNA damage and repair.

4.1. Modifications of the comet assay

As mentioned in the previous section, the standard version of the comet assay detects both DNA SBs and ALS. However, with some modifications, a wider scope of lesions can be detected.

First of all, digestion of nucleoids with lesion-specific enzymes from the bacterial and human DNA repair machinery, allows the detection of altered (*e.g.*, oxidised or alkylated) bases. These enzymes induce (additional) breaks in the sites of these altered bases which are easily measured following the rest of the assay's protocol. Formamidopyrimidine-DNA glycosylase (Fpg), which detects a variety of DNA lesions including oxidised purines (Table II), is the most frequently used. Figure 6 shows a scheme of the standard comet assay protocol and the modified assay including digestion with lesion-specific enzymes (Fpg or Endocuclease III) to detect oxidised bases. Examples of different DNA repair enzymes which have been already used in combination with the comet assay are provided in Table II. The majority of them have been extensively used in the context of human biomonitoring (Azqueta and Collins, 2013).

Table II. Examples of human and bacterial DNA repair enzymes used in combination with the comet assay.

Enzyme; Enzyme Commission (EC) Number	Origin	Type of lesion(s) detected	Reference
Endonuclease III (Endo III); EC 4.2.99.18	Bacterial	Oxidised pyrimidines	Collins *et al.*, 1993
Formamidopyrimidine-DNA glycosylase (Fpg); EC 3.2.2.23	Bacterial	Oxidised purines (8-oxoGua*), ring-opened purines and ring-opened N7 guanine adducts	Dusinska and Collins, 1996
8-oxoguanine DNA glycosylase (OGG1); EC 4.2.99.18	Human	8-oxoGua	Smith *et al.*, 2006
3-methyladenine glycosylase II (Alk A); EC 3.2.2.21	Bacterial	3-methyladenine	Berdal *et al.*, 1998
Uracil DNA glycosylase (UDG); EC 3.2.2.3	Human	Misincorporated uracil	Duthie and McMillan, 1997
Pyrimidine dimer DNA glycosylase (T4 endonuclease V); EC 3.1.25.1	Bacterial	UV-induced dimerised pyrimidines	Collins *et al.*, 1997

*8-oxo-7,8-dihydroguanine (8-oxoGua)

Using the enzymes in combination with the comet assay seems to be a very useful tool in genotoxicity testing since other DNA lesions apart from SBs can be detected. However, their specificity has not been thoroughly studied in all the cases yet. For example, apart from detecting oxidative DNA damage, Fpg also attacks ring-opened N7 guanine adducts produced by alkylating agents (Li *et al.*, 1997; Speit *et al.*, 2004). Nevertheless, Azqueta *et al.* (2013a) proved that Fpg enhanced the sensitivity of the *in vitro* alkaline comet assay in genotoxicity testing without losing its selectivity by testing 11 chemicals in TK6 cells.

Other modifications of the comet assay allow the measurement of DNA-DNA cross-links (*e.g.*, Spanswick *et al.*, 2010), DNA-protein cross-links (*e.g.*, Tice *et al.*, 2000), DNA repair activity (reviewed in Azqueta *et al.*, 2014) and even global methylation (Georgieva *et al.*, 2017; Lewies *et al.*, 2014; Wentzel *et al.*, 2010). Moreover, the combination of the comet assay with fluorescent in situ hybridization (FISH) has been used to detect specific genes (reviewed in Shaposhnikov *et al.*, 2009). It is worth to mention that all these modifications have been used in human biomonitoring or in basic research but their application in genotoxicity testing, if relevant, has not been explored.

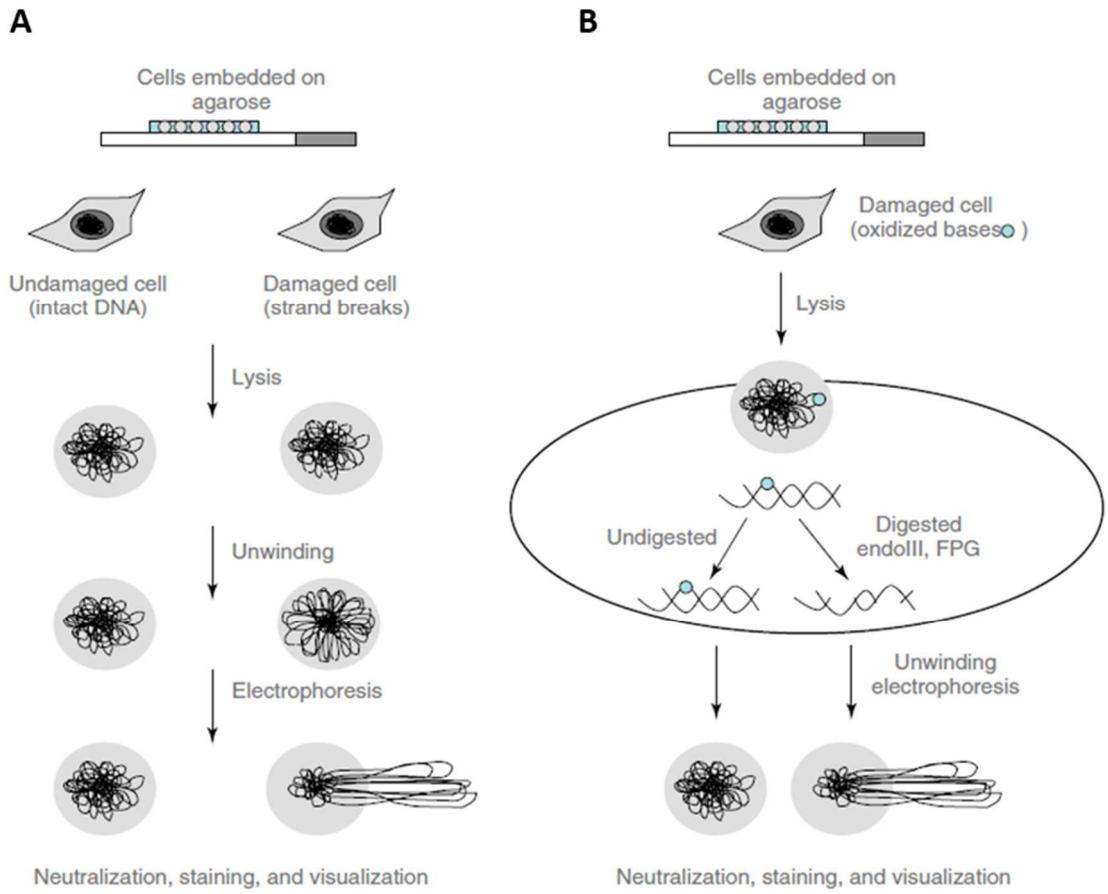

Figure 6. Scheme of the standard comet assay (A), and the modified assay including digestion with lesion-specific enzymes to detect oxidised bases (B). From Azqueta and Collins, 2011.

4.2. The dark side of the comet assay and some good news

Scoring comets is a tedious task that implies a lot of hours at the microscope. Automated scoring methods have been developed by different companies (*e.g.*, Imstar, Metasystems). They are very useful tools, under continuous improvements, though still not always very accurate in detecting and analysing all comets. In any case, the unaffordable prices of these systems make it almost impossible to use them at the research level.

On the other hand, the efficiency of the traditional version of the assay, in which 1, 2 or 3 large gels are placed on a microscope slide, is rather low due to the limited number of samples that can be processed in one experiment (normally determined by the number of slides that can be placed on a conventional electrophoresis tank). However, several authors have developed their own medium and high-throughput methods (reviewed in Brunborg *et al.*, 2014), some of which are very easy to implement (*e.g.*, placing multiple mini-gels on top of microscope slides

or plastic GelBond® Films), and only slight modifications of the protocol are needed (Gutzkow *et al.*, 2013; Mcnamee *et al.*, 2000; Shaposhnikov *et al.*, 2010). Validation of the medium- and high-throughput methods against the traditional version has been carried out by different authors, showing that similar results are obtained (Azqueta *et al.*, 2013b). By applying these methods, the cost and time to process samples are reduced, but the number of samples to score increases substantially and thus the development of low-cost automated scoring systems is urgently required.

From the earliest papers, it was noticed that the variability in the application of the technique itself consistently difficulted inter-laboratory comparison of results (Fairbairn *et al.*, 1995); that is to say, differences in the protocols used by different research groups made it hard to compare results between laboratories. Relatively high inter-laboratory variation has been reported in various studies (ESCODD, 200; Ersson *et al.*, 2013; Forchhammer *et al.*, 2012, 2010; Johansson *et al.*, 2010; Møller *et al.*, 2010). Moreover, quite high inter-experimental variation (Azqueta and Collins, 2013 and Møller *et al.*, 2010) as well as intra-assay variation has been also reported (Gutzkow *et al.*, 2013 and Møller *et al.*, 2010). As it is explained in the next section, a lot of effort has been done in order to reduce the variation of the comet assay.

4.3. Standardisation of the assay

An expert panel was convened at the International Workshop on Genotoxicity Test Procedures (IWGTP), held in in Washington in March 1999, in order to identify minimal standards for obtaining reproducible and reliable comet assay data deemed suitable for regulatory submission. They reached the consensus that the alkaline comet assay was optimal for identifying agents with genotoxic activity. In addition, the critical technical steps of both the *in vitro* and *in vivo* versions of the assay were discussed, and guidelines developed (Tice *et al.*, 2000). More detailed practical guidance to conduct the *in vivo* comet assay for regulatory purposes was published later (Hartmann *et al.*, 2003a).

Some important factors influencing the outcome of the comet assay have been thoroughly studied to date. Final agarose concentration in gels is inversely proportional to the levels of DNA damage detected in treated cells (Azqueta *et al.*, 2011; Ersson and Möller, 2011). On the other hand, increasing the duration of the alkaline treatment (Azqueta *et al.*, 2011; Ersson and Möller, 2011; Speit *et al.*, 1999; Vijayalaxmi *et al.*, 1992; Yendle *et al.*, 1997) and electrophoresis (Azqueta *et al.*, 2011; Ersson and Möller, 2011; Speit *et al.*, 1999; Vijayalaxmi *et al.*, 1992), as well as the voltage applied in the electrophoresis tank (Azqueta *et al.*, 2011; Ersson and Möller, 2011), have shown to increase the extent of DNA damage measured.

Scoring is also considered a critical step. It has been demonstrated that different concentrations of dye also affect the assay's sensitivity to detect DNA damage (Olive *et al.*, 1990). Besides, microscope quality and adjustments, aging of the UV lamp and settings within the image analysis software also affect the results.

When using the Fpg-modified assay, both the duration of the alkaline and enzyme incubations has been shown to affect the detection of enzyme-sensitive sites (Ersson and Möller, 2011).

Recently, general recommendations to carry out the comet assay taking into account those critical factors were published (Azqueta and Collins, 2013). Moreover, an OECD Guideline to carry out the *in vivo* comet assay was published in 2014 (OECD, 2016a) (see section 5.1.1).

4.4. Reducing variability

In an inter-laboratory study carried out by 14 participating laboratories, Ersson *et al.* (2013) assessed the inter- and intra-laboratory, sample and residual variations in DNA SBs and Fpg-sensitive sites measured with the comet assay in coded peripheral blood mononuclear cells (PBMC); what they found was that inter-laboratory variation accounted for the largest fraction of the overall variation. Godschalk *et al.* (2014) came to the same conclusion in another inter-laboratory-variation study involving 13 different laboratories. Though standardisation of the assay and the development of standard protocols are crucial for controlling data variation, adherence to a standard protocol only slightly reduced the inter-laboratory variation in Fpg-sensitive sites detected in human mononuclear blood cells (MNBCs) (Forchhammer *et al.*, 2012). In addition, comet scoring does not entirely depend on the comet assay protocol applied; as it has been mentioned before, results depend on the microscope quality and adjustments, the aging of the UV lamp and the settings within the image analysis software.

Inclusion of reference standards (*i.e.*, cells with a known amount of specific DNA damage) or normalisation of results using a calibration curve (usually performed with X-ray-treated cells) have been proposed as valuable tools to reduce the inter-experimental variation (Azqueta and Collins, 2013; Collins *et al.*, 2014; Zainol *et al.*, 2009). Moreover, the latter approach led to a statistically significant reduction in the coefficient of variation (CV, from 47% to 28%) of the DNA damage obtained by 12 different laboratories in irradiated monocyte-derived THP-1 cells (Forchhammer *et al.*, 2010). A similar approach was used to reduce the variation in the Fpg-sensitive sites of X-irradiated A549 lung epithelial cells estimated by different investigators (Møller *et al.*, 2004), in the oxidised purines detected in MNBCs exposed to ɣ-radiation (Forchhammer *et al.*, 2008), and the inter-laboratory variation found between 10 laboratories

in the measurement of Fpg-sensitive sites, which was mainly due to protocol differences (Johansson *et al.*, 2010).

Last but not least, controlled electrophoresis (including circulation of the electrophoresis solution) has been shown to improve the homogeneity between replicate samples (*i.e.*, intra-assay variability) in a 96-mini-gel format (Gutzkow *et al.*, 2013) and will probably reduce the inter-experimental variability.

The European Standards Committee on Oxidative DNA Damage (ESCODD) sent photosensitiser plus light-treated HeLa cells to compare the levels of 8-oxoGua measured with the Fpg-modified comet assay to 10 different laboratories (ESCODD, 2003). Among them, only 5 were able to detect a dose-response. In another study (Gedik and Collins, 2005), they found a 10-fold variation in the Fpg-sensitive sites of control HeLa cells measured in 8 different laboratories (six of them followed the same protocol).

Notwithstanding the identification of several critical assay parameters, and the numerous inter-laboratory trials carried out by the European Standards Committee on Oxidative DNA Damage (ESCODD, 2003; Gedik and Collins, 2005) and, primarily, by the European Comet Assay Validation Group (ECVAG; Ersson *et al.*, 2013; Forchhammer *et al.*, 2012, 2010; Godschalk *et al.*, 2014; Johansson *et al.*, 2010) to both assess the variability and try to control or even decrease it, further studies are needed as to definitely eliminate variability from the comet assay, notably the one added in the staining, scoring and image analysis steps (Collins *et al.*, 2014).

5. The comet assay in regulatory (geno)toxicology

Although the comet assay has been extensively used for almost 30 years now, its role in regulatory genotoxicology was rather limited. This might be probably due to the fact that the standard alkaline comet assay detects DNA SBs (and ALS), which are quickly repaired and not very relevant in terms of genetic stability. Moreover, other *in vitro* assays detecting gene mutations and structural and numerical chromosome aberrations were already available. In addition, the high inter-experimental and inter-laboratory variation did not play in its favour.

However, a great advantage of the comet assay is that it can be applied to any organ as long as a cell suspension can be obtained, and in non-dividing cells; this characteristic makes it very useful in testing *in vivo* genotoxicity since the majority of the aforementioned assays are applied in blood or bone marrow cells (*i.e.*, dividing cells). During the last years, both the *in*

vivo and the *in vitro* versions of the comet assay have made their way into regulatory toxicology.

5.1. The *in vivo* comet assay

5.1.1. OECD Guideline

Already in 2004, the *in vivo* comet assay was suggested to be used as a complementary assay for mechanistic investigations following positive *in vitro* findings and to evaluate target organ-specific genotoxicity for the critical risk assessment within the regulatory acceptance of pharmaceuticals (Hartmann *et al.*, 2004). Guidelines and recommendations were published all over the world by scientific committees and regulatory agencies such as the Committee on Mutagenicity of Chemicals in Food, Consumer products and the Environment (COM), the U.S. Food and Drug Administration Centre for Drug Evaluation and Research (FDA-CDER) and the European Medicines Agency (EMA) (reviewed in Cimino, 2006). During the 4th International Comet Assay Workshop on Genotoxicity Testing (IWGT), held in San Francisco (California) in September 2005, some of the procedures and methods recommended to carry out the *in vivo* rodent alkaline comet assay were discussed in order to maximize its acceptance for regulatory purposes (Burlinson *et al.*, 2007), while critical topics related to its use in regulatory genotoxicity testing were evaluated by an expert working group during the 6th IWGT, held in Foz do Iguacu (Brazil) in October/November 2013 (Speit *et al.*, 2015).

Although international agreed protocols were available, it was not until the first formal validation trial, coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the U.S. NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM), and the Japanese Environmental Mutagen Society/Mammalian Mutagenesis Study Group (JEMS/MMS) (Uno *et al.*, 2015a, 2015b), that an *In vivo* Mammalian Alkaline Comet Assay OECD (TG 489) Guideline was achieved. The first version was approved in 2014, while the last one was adopted on 29th July 2016 (OECD, 2016a). In line with the 'three Rs' (Reduce, Refine and Replace) principles, the Guideline considers the integration of the comet assay into repeated-dose toxicity studies, or its combination with other genotoxicity endpoints such as the micronucleus assay.

The guideline establishes common laboratory strains of healthy young adult rodents (6-10 weeks old) to be used. Regarding the experimental design, a minimum of 5 analysable animals

of one sex (or of both sexes if there is existing data demonstrating relevant sex-differences) should be used. For both acute and sub-acute versions of the comet assay, the maximum tolerated dose (MTD), together with a descending sequence of at least two additional appropriately spaced dose levels should be selected, covering a range from the maximum to little or no toxicity. For a non-toxic test chemical, the Guideline provides maximum (limit) doses depending on the administration period. Animals should be treated daily over 2 or more days, and samples should be collected once at 2-6 h after the last treatment in order not to miss transient DNA lesions. As no inter-laboratory studies have been conducted in tissues other than liver and stomach, no recommendations about optimum or acceptable ranges for other tissues are available (the group mean % tail DNA should not exceed 6% for rat liver). However, the Guideline mentions several other tissues to which the technique has been applied. For each sample, the Guideline establishes that 150 cells should be analysed at least, being the % tail DNA recommended for the interpretation of results. Regarding the interpretation of results, a test chemical is considered able to induce DNA strand breakage if the 3 following criteria are met: at least one of the test doses exhibits a statistically significant increase compared with the negative control, the increase is dose-related, and any of the results are outside the distribution of the historical negative control data for the given experimental conditions. However, it is emphasised that tissue cytotoxicity might result in an increased DNA migration, for which examination of histopathological changes is considered a relevant measure. Anyway, a careful interpretation of results should be done in the presence of clear signs of cytotoxicity.

As a result of the final validation study, the comet assay was considered highly capable of identifying genotoxic chemicals (at least in liver and stomach, although other tissues could be used), and therefore could serve as a reliable predictor of rodent carcinogenicity (Uno *et al.*, 2015b). However, as inter-laboratory studies have been only carried out in liver and stomach, no recommendation has been established for how to achieve a sensitive and reproducible response in other tissues, such as expected positive and negative control ranges. In addition, the Guideline recognises the possibility of using frozen tissues, as long as the laboratory demonstrates its competency in freezing methodologies, confirms acceptable low ranges of % tail DNA in target tissues of vehicle treated animals and the detection of positive responses. Regarding the use of lesion-specific enzymes or other modifications of the assay to detect other types of DNA lesions, the Guideline states that further work would be needed to characterize the necessary protocol modifications.

Nowadays, the *in vivo* comet assay is part of the strategy suggested by the ICH (ICH, 2012) and it is also contemplated by the European Food Safety Authority for the genotoxicity testing of compounds in food and animal feedstuffs (EFSA, 2011) (see section 2). Overall, the number of applications containing *in vivo* comet assay data has increased since 2007, and this trend is expected to be sustained (Frötschl, 2015).

5.1.2. Combination of the *in vivo* comet assay with other assays

Already in 1991, it was raised that *in vivo* genotoxicity tests could be combined with other toxicological investigations (*e.g.*, within the framework of 28-day tolerance studies) (Fahrig *et al.*, 1991). During the 5th International Workshop on Genotoxicity Testing (IWGT), held in Basel (Switzerland) in August 2009, a working group discussed how to improve *in vivo* genotoxicity assessment, while, at the same time, trying to implement the 'three Rs' concept. Due to the low sensitivity of the liver unscheduled DNA synthesis (UDS) test in the detection of rodent carcinogens which were not detected with the *in vivo* micronucleus test (they both were traditionally used as *in vivo* follow-up tests), other methods were searched as possible replacements for that endpoint. In this context, the working group discussed and finally agreed that it was technically feasible and scientifically acceptable to combine and integrate the *in vivo* micronucleus and liver comet assays for both acute and repeated dose studies (Rothfuss *et al.*, 2011, 2010). The technical feasibility and complementary use of different target organs and genetic endpoints, in addition to similar experimental requirements, strongly support the combination of these two assays.

During the 6th IWGT (held in Brazil in 2013), it was corroborated that integration of the comet assay into a repeated-dose toxicity study, as well as the combination of the comet assay with an acute MN assay are possible (Speit *et al.*, 2015), which had been already discussed as available and scientifically credible reduction options in the number of animals at the ECVAM workshop held in Italy in June 2008 (Pfuhler *et al.*, 2009). In fact, the NTP uses the second approach to evaluate the genotoxicity of substances of public concern (Recio *et al.*, 2010). Moreover, the ICH guidelines for genotoxicity testing (ICH, 2012) and the Integrated Testing Strategy (ITS) of the Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH) programme of the European Commission (ECHA, 2017) also promote the integration of genotoxicity tests into repeated-dose toxicity studies.

5.1.3. The *in vivo* comet assay *versus* other genotoxicity assays

Sasaki *et al.* (2000) found that 49 out of 54 rodent carcinogens that did not induce micronuclei were positive in the comet assay, suggesting that it could be used as a further *in vivo* test apart from the cytogenetic assays in hematopoietic cells. However, this paper has been criticised because it does not fully meet the requirements for an acceptable test agreed during the IWGTP meeting in Washington in 1999 (Tice *et al.*, 2000). In the same vein, the ability of the *in vivo* UDS, transgenic mutation and comet assays to detect rodent carcinogens which gave either negative or equivocal results in the bone-marrow MN test has been compared (Kirkland and Speit, 2008). Among them, the comet assay was found to be the most sensitive test (almost 90% of the micronucleus-negative or equivocal carcinogens were detected), with an acceptable specificity (78% negative results with non-carcinogens). Finally, in a collaborative trial (Rothfuss *et al.*, 2010), another group of experts reached the conclusion that the liver comet assay (using either a short- or a long-term protocol) was a reasonable alternative to the UDS test.

5.2. The *in vitro* comet assay

5.2.1. Validation of the *in vitro* comet assay

In vitro genotoxicity tests in mammalian cells produce a remarkably high and unacceptable occurrence of irrelevant positive results; thus, better guidance on the likely mechanisms involved, and how to obtain evidence for those mechanisms, is needed (Kirkland *et al.*, 2007). In fact, improving current *in vitro* tests in order to reduce false positives and to avoid unnecessary *in vivo* follow-up tests, present major challenges for genetic toxicologists (Pfuhler *et al.*, 2011, 2009).

However, it is known that a great part of the false positives reported by the comet assay, and by other *in vitro* genotoxicity assays, are caused by testing cytotoxic concentrations of the compounds (*i.e.*, cell death causes secondary DNA that may lead to the incorrect positive classification of a chemical) (Fowler *et al.*, 2012). In the *in vitro* comet assay, it is recommended to avoid concentrations of a test chemical producing high (>30%) mortality (Tice *et al.*, 2000). In this context, cell proliferation assays are the best measures of cytotoxicity (Fowler *et al.*, 2012; Kirkland, 2011).

When evaluating the suitability of the comet assay as a screening test during industrial drug development, a high degree of concordance was found between the results of the *in vitro* comet assay and the *in vitro* MN test by analysing 36 pharmaceutical compounds with

unknown genotoxic potential (Hartmann *et al.*, 2001). In another study, Hartmann *et al.* (2003b) found a high degree of agreement between the *in vitro* comet assay and the chromosome aberration test for 13 drug candidates. Furthermore, the sensitivity of the *in vitro* comet assay has been shown to be similar than the one of the micronucleus, chromosome aberration and sister chromatid exchange assays (Uhl *et al.*, 1999). Nonetheless, the *in vitro* comet assay is not regarded as a standard battery test, and it is rarely submitted for regulatory purposes (Frötschl, 2015).

Nowadays, the *in vitro* version of the alkaline comet assay is widely used for genotoxicity screening of novel cosmetics, nanomaterials and pharmaceuticals, and it is also recommended as an appropriate test for use under the REACH programme of the European Commission (ECHA, 2017). However, the initiative implemented by EURL-ECVAM to evaluate the validity of the *in vitro* comet assay (Burlinson *et al.*, 2007) is currently stopped (EURL-ECVAM, 2014).

5.2.2. The *in vitro* comet assay in 3D skin models

The prohibition of animal testing for cosmetic ingredients as from March 2013 (Regulation 1223/2009), together with the availability of *in vitro* models adequately reproducing human skin, position the *in vitro* comet assay as a good option in this area.

During the 5th IWGT (Rothfuss *et al.*, 2011), the results of a project sponsored by the former European Cosmetics Industry Association (COLIPA), with a contribution from ECVAM, were presented. In addition to the pre-validation studies of the *in vitro* reconstructed skin micronucleus (RSMN) assay, a good inter-laboratory reproducibility of the 3D skin comet assay was demonstrated for the direct-acting mutagens methyl methanesulfonate (MMS) and 4-nitroquinoline 1-oxide using three different real skin models.
Nowadays, the validation study, coordinated by Cosmetics Europe, in collaboration with the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM), is still ongoing (EURL-ECVAM, 2014).

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Chapter 2

Aim, objectives and outline

1. AIM

It seems clear that both the *in vivo* and *in vitro* versions of the comet assay have a wide range of applications, and nobody ventures to discuss their usefulness. However, the trip for the standardisation of the assay has not been easy. Indeed, it is not yet completed.

1.1. *In vitro* comet assay

As mentioned in the introduction, several critical points affecting the outcome of the alkaline comet assay have been already identified. Lysis conditions are considered a critical variable that may interfere with the outcome resulting from specific types of DNA modifications (certain DNA alkylations and base adducts). Thereby, it is recommended to keep lysis conditions as constant as possible for all the slides within an experiment (from 1 hour to overnight lysis at around 2-8°C under subdued lighting conditions). However, the influence of lysis conditions (*i.e.*, lysis duration and composition of the lysis solution) has not been thoroughly studied.

1.2. *In vivo* comet assay

Taking the "Initial considerations and limitations" section of the *In vivo* Mammalian Alkaline Comet Assay OECD (TG 489) Guideline as a reference, someone can easily detect improvement points of particular interest.

The first one has to do with the tissues to which the comet assay can be applied. Although the Guideline recognises that the technique is in principle applicable to any tissue from which analysable single cell/nuclei suspensions can be derived, it encourages the laboratory to demonstrate proficiency with each individual tissue in each species they are planning to study, and that an acceptable positive response with a known mutagen can be obtained in that tissue. The group mean % tail DNA in the rat liver should not exceed 6%, although it does not give recommendations about acceptable ranges for other tissues. As inter-laboratory studies have been only carried out in liver and stomach, no recommendation has been established for how to achieve a sensitive and reproducible response in other tissues

The second one concerns the chance to freeze tissues or cell nuclei for later analysis, which has been already successfully described, usually resulting in a measurable effect on the response to the vehicle and positive control. However, the Guideline suggests that, if used, the laboratory should demonstrate competency in freezing methodologies and confirm acceptable low ranges of % tail DNA in target tissues of vehicle treated animals, as well as being able to

detect positive responses. In addition, although the freezing of tissues has been described using different methods, currently there is no agreement on the best way to freeze and thaw tissues.

In the same vein, another two questions come to mind: Is it possible to apply the Fpg-modified assay to frozen tissue samples? And, for how long can tissue samples be frozen for later comet analysis? With regard to the first one, this possibility is not even contemplated in the *in vivo* OECD Guideline; although it recognises that oxidised bases might be detected, the necessary protocol modifications are supposedly not still adequately characterised. With regard to the second one, according to our knowledge, there is no article specifically studying the long-term stability of frozen tissue samples, another key issue.

2. Objectives and outline

Therefore, the aim of the present project is to contribute to the standardisation of the *in vitro* and *in vivo* comet assays by providing knowledge of their critical points, and therefore to increase their applicability from a regulatory point of view.

Objectives:

1. To study the influence of the time of lysis and lysis solution composition in the *in vitro* alkaline comet assay results.
   For this purpose, two different approaches were used:
   1.1. Different times of lysis (from no lysis to 1 week) were tested in HeLa cells untreated or treated with compounds able to induce alkylated bases or oxidative DNA damage: methyl methanesulfonate (MMS), H_2O_2 or Ro 19-8022 + light (the Fpg-modified assay was used in the last case). **Chapter 3.**
   1.2. Different times of lysis (from no lysis to 1 week) and two different lysis solutions were tested in control and X-ray-treated TK6 cells, which mainly induces DNA single strand breaks. **Chapter 4.**
2. To study the best freezing/thawing method for the tissue samples in order to apply the *in vivo* alkaline comet assay with/without Fpg. **Chapter 5.**
   For this purpose, a step-wise approach was followed:
   2.1. First of all, different freezing/thawing methods were tested in frozen liver tissue samples from untreated animals to select the best one.
   2.2. Secondly, the best method was used to compare the DNA damage detected in fresh and frozen (for different time-lengths) liver, kidney and lung tissue samples from rats

orally administered with different concentrations of MMS. Again, both the standard and the Fpg-modified comet assays were used.

3. To apply the *in vivo* alkaline comet assay to frozen kidney tissue samples obtained in a previous repeated-dose toxicity study of the food contaminant ochratoxin A (OTA). For this purpose, samples from male and female F344 rats orally administered with different doses of OTA for 7 or 21 days were analysed. Furthermore, oxidative DNA damage was phenotypically collated by determining glutathione S-transferase (GST) activity, total (tGSH) and oxidised (GSSG) glutathione levels and superoxide dismutase (SOD) activity in kidney tissue samples from the same animals. **Chapter 6.**
4. To collaborate in the elaboration of a Standard Operating Procedure (SOP) for the *in vitro* alkaline comet assay, and to elaborate a SOP for the *in vivo* alkaline comet assay performed either in fresh or frozen tissue, to be later applied in genotoxicology studies performed under Good Laboratory Practice (GLP) conditions.

Chapter 3

Does the duration of lysis affect the sensitivity of the *in vitro* alkaline comet assay?

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Q2 in "Toxicology" (JCR, 2015)

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Chapter 4

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

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Chapter 5

Applying the comet assay to fresh *vs.* frozen animal solid tissues: a technical approach

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Abstract

The *in vivo* comet assay is usually performed in fresh tissues by processing cells immediately after collection, an approach that is not always possible from a logistical point of view. Although the comet assay has been applied to frozen rodent tissue samples on several occasions, there is currently no agreement on the best way to freeze and thaw them. Furthermore, the *In vivo* Mammalian Alkaline Comet Assay OECD (TG 489) Guideline requires the demonstration of the laboratory's proficiency in freezing methodologies. In this regard, we have not only tested different freezing/thawing procedures in liver tissues from untreated rats, but we have also compared the levels of DNA strand breaks and Fpg-sensitive sites between fresh and 1-week or 1-month frozen liver, kidney and lung tissue samples from untreated and MMS-treated rats. Among the five different procedures tested, only one approach gave acceptable results, leading to the conclusion that the thawing process is equally or even more determinant than the freezing one in the preservation of DNA integrity. Using this approach, our results show that comparable levels of SBs and net Fpg-sensitive sites are detected either in fresh or in frozen liver, kidney and lung tissue samples.

1. Introduction

The single-cell gel electrophoresis (comet) assay is a genotoxicity assay which, due to its various advantages, has been widely used in several areas, such as *in vitro* and *in vivo* genotoxicity testing.

As a result of the first formal validation trial, coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) between 2006-2012 (Uno *et al.*, 2015a, 2015b), an *In vivo* Mammalian Alkaline Comet Assay OECD Guideline was achieved (the first version was approved in 2014, while the last one was adopted on 29th July 2016) (OECD, 2016). Nowadays, the assay is part of the strategy suggested by the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (ICH, 2012) and it is also contemplated by the European Food Safety Authority (EFSA) for the genotoxicity testing of compounds in food and animal feedstuffs (EFSA, 2011).

The *in vivo* comet assay is usually performed in fresh tissues by processing cells immediately after collection, an approach that is not always possible from a logistical point of view due to the high number of samples that need to be handled (Brunborg *et al.*, 2014; Pant *et al.*, 2014). Moreover, it is advisable to integrate the *in vivo* comet assay into repeated-dose toxicity studies (Recio *et al.*, 2012; Rothfuss *et al.*, 2011) or to perform it in combination with the micronucleus assay (Recio *et al.*, 2010). Thereby, freezing of tissues for later analysis emerged as an option to overcome the logistic problems. Although the OECD guideline recognises that tissues or cell nuclei have been successfully frozen for later analysis, it also requires the demonstration of the laboratory's proficiency in freezing methodologies (OECD, 2016).

In the literature, the comet assay has been applied to several frozen rodent tissue samples as liver (*e.g.*, Folkmann *et al.*, 2007; Jackson *et al.*, 2013; Knudsen *et al.*, 2015; Løhr *et al.*, 2015; Risom *et al.*, 2007), kidney (*e.g.*, Knudsen *et al.*, 2015), lung (*e.g.*, Folkmann *et al.*, 2007; Jackson *et al.*, 2013; Knudsen *et al.*, 2015; Risom *et al.*, 2007), brain (*e.g.*, Forsberg *et al.*, 2015; Knudsen *et al.*, 2015) and spleen (*e.g.*, Knudsen *et al.*, 2015). Moreover, all these studies performed the comet assay in combination with enzymes (*i.e.*, Formamidopyrimidine-DNA glycosylase, Fpg; Endonuclease III, Endo III; or 8-oxoguanine DNA glycosylase, OGG1).

According to the OECD Guideline, there is currently no agreement on the best way to freeze and thaw tissues (OECD, 2016). Small tissue samples are most commonly snap frozen in liquid nitrogen and stored at -80°C until further processing (Folkmann *et al.*, 2007; Forsberg *et al.*, 2015; Jackson *et al.*, 2013; Knudsen *et al.*, 2015; Løhr *et al.*, 2015; Risom *et al.*, 2007).

However, several authors freeze tissue samples as cell suspensions using DMSO as a cryoprotectant (Hu *et al.*, 2002; kraynak *et al.*, 2015; Pant *et al.*, 2014; Recio *et al.*, 2010).

According to our knowledge, Jackson and colleagues have been the only group that has thoroughly described both the freezing and thawing process (including the preparation of cell suspensions from frozen tissues) (Jackson *et al.*, 2013). Actually, they tested four different freezing/thawing methods for liver samples from untreated mice. Their results showed that snap freezing in liquid nitrogen of small pieces (*i.e.*, 3 x 3 x 3 mm) of tissue (previously placed in cryotubes), in combination with the disgregation of the deep-frozen tissue (*i.e.*, avoiding it to thaw) in ice-cold Merchant's medium using a metal sieve, gave very low levels of DNA strand breaks (SBs). On the other hand, leaving the samples to thaw at room temperature yielded very high levels of DNA SBs. Moreover, this is the only work in which the comparison between fresh and immediately frozen tissues has been done; specifically, they compared the results obtained with the standard comet assay in fresh and frozen liver and lung tissues from mice treated intraperitoneally with different concentrations of methyl methanesulfonate (MMS). Results showed a high correlation between DNA damage and MMS concentration. Overall, no significant differences were observed between fresh and frozen tissues, except a significant slight increase of the % tail DNA in frozen lung tissue from untreated animals in comparison with the freshly-prepared one (from 2.9 to 7.1%).

In this work, we have explored different freezing/thawing methods to analyse their impact not only on the level of DNA SBs, but also on the Fpg-sensitive sites (*i.e.*, oxidised and alkylated bases) detected in liver tissues from untreated rats. In addition, we have compared the levels of these lesions in fresh and frozen liver, kidney and lung tissues from rats exposed to different concentrations of MMS.

2. Materials and methods

2.1. Chemicals and reagents

Low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, BSA, MMS and 4',6-diamidino-2-phenylindole (DAPI) (Item No. D9542, Sigma) were purchased from Sigma-Aldrich. NaCl, NaOH, Na_2HPO_4, KH_2PO_4 and KCl were purchased from PanReac AppliChem and 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. Fpg was a gift from NorGenoTech (Norway).

2.2. Animals

For step 1 (see next section), in which liver tissues were analysed, untreated rats from other toxicological studies were used. These studies were approved by the Ethics Committee on Animal Experimentation of the University of Navarra. The use of this material is in agreement with the 'three Rs' (Reduce, Refine and Replace) strategy for experimental animals.

For step 2 (see next section), fifteen male Wistar rats, 8 weeks-old, were purchased from Charles River. Animals were randomly distributed in groups of five animals per cage and used after 1 week of acclimatization under standard conditions (temperature 22 ± 3°C, humidity 50 ± 20%, 12 h light/dark cycle). Animals were fed with standard laboratory chow and allowed to access *ad libitum* feed and drinking water.

Each day, one animal of each group was orally administered 5 or 200 mg/kg b.w. of MMS, or nothing (negative control group). Three hours after the administration, animals were anesthetised with isoflurane, sacrificed by cervical dislocation and their liver, kidneys and lungs were removed and processed as described below.

This study was approved by the Ethics Committee on Animal Experimentation of the University of Navarra.

2.3. Sample processing

The study was performed following a two-step process. The aim of step one was to select the best freezing/thawing procedure using liver samples from untreated rats. In step 2, the best procedure was applied to liver, kidney and lung samples from untreated and MMS-treated rats and a stability study of the frozen samples was performed.

2.3.1. Step 1

For step 1, 3 x 3 x 3 mm liver pieces from untreated rats were obtained directly at necropsy by cutting the liver on an ice-cold Petri's dish. Five different approaches were followed by combining different freezing/thawing procedures (Figure 1). Regarding the freezing process, 4 procedures were followed: 1.- flash freezing in liquid nitrogen after transferring them into cryotubes and storage at -80°C (approaches 1 and 2); 2.- flash freezing in isopentane cooled in liquid nitrogen after transferring them into cryotubes and storage at -80 °C (approach 3); 3.- overnight immersion in 4°C Merchant's medium (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) , transferring to an empty bijou on ice and storage

at -80°C (approach 4); 4.- overnight immersion in 4°C RNA later® solution, transferring to an empty bijou on ice and storage at -80°C (approach 5).

For the thawing process, 2 different procedures were used. The first one was used as part of approach 1. Cryotubes were placed on dry ice and a drop of Merchant's medium was placed on the tissue to create a protective ice cap. The deep-frozen tissue was transferred into the cylindrical metal sieve, previously immersed in 3 mL ice-cold Merchant´s medium, using cold tweezers and homogenised by moving a plastic plunger up and down several times, forcing it to pass through the sieves. In this way, a cellular suspension was obtained and left on ice until comet assay analysis. During all this process samples were kept on ice.

The second thawing procedure was used as part of approaches 2, 3, 4 and 5: frozen samples were immediately put on ice, washed in a beaker with ice-cold Merchant's medium, transferred to another beaker with ice-cold Merchant's medium, cut into pieces with scissors, homogenised using a cylindrical metal sieve (*i.e.*, a stainless steel cylindrical tube of 15 mm diameter with a stainless-steel screen of 0.4 mm fitted inside) by moving a plastic plunger up and down several times, filtered through a 100 µm nylon filter, centrifuged for 5 minutes at 214 x g and 4°C and resuspended in Merchant's medium (centrifuged twice) .

Several samples were analysed using approach number 1.

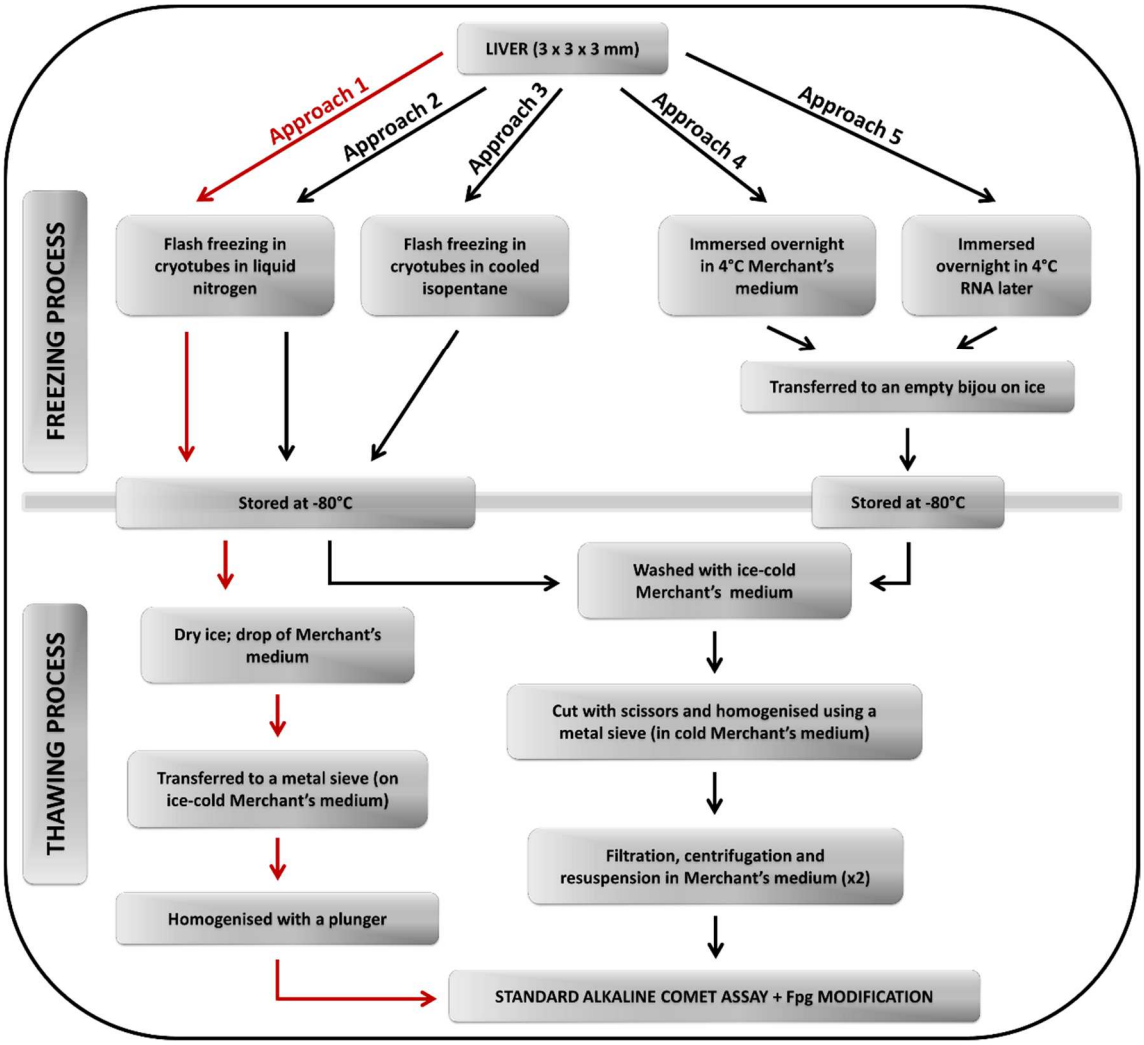

Figure 1. Schematic diagram of the different freezing and thawing procedures tested in liver samples.

2.3.2. Step 2

Fresh and frozen liver, kidney and lung tissue samples from rats administered orally with 5 or 200 mg/kg b.w. of MMS, and non-administered ones were used. One animal of each group was administered and sampled each day of the week and animals were sacrificed 3 hours after the administration.

The freezing and thawing procedures were performed using the successful approach from step 1 (approach 1). Frozen samples were processed after 1 week and 1 month. (Frozen samples after 3 months, 6 months and 1 year are under currently analysis; results will be included in the manuscript before its submission).

The standard comet assay was applied to fresh and frozen liver, kidney and lung tissue samples from animals of the 3 different groups (*i.e.*, untreated rats, rats treated with 5 mg/kg b.w. of

MMS, and rats treated with 200 mg/kg b.w. of MMS). In addition, the comet assay in combination with Fpg was applied to fresh and frozen liver, kidney and lung tissue samples from rats either untreated or treated with 5 mg/kg b.w. of MMS.

2.4. Comet assay

Thirty microliters of the cellular suspension of each sample were mixed with 140 µL of 1% low melting point agarose in PBS at 37°C. Immediately, two drops of 70 µL each were placed on a glass microscope slide (pre-coated with 1% normal melting point agarose in distilled water and dried) and covered with 20 x 20 mm coverslips. Gels were set on a metal plate on ice for 3 min and the coverslips were removed. Three slides were prepared per condition: 'lysis', 'buffer' and 'Fpg'.

Overnight lysis at 4 °C was performed by immersion in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Trizma® base, pH 10.0, 1% Triton X-100). After lysis, 'Fpg' and 'Buffer F' slides were washed three times (5 min each) with the Fpg reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0—Buffer F) at 4°C. Afterwards, gels were incubated with Buffer F or Fpg by adding a drop of 45 µL of the solutions on top of the corresponding ones. Each drop was covered with a 22 x 22 mm coverslip and the gels were incubated in a humidified atmosphere at 37°C for 30 min. 'Lysis' slides were kept immersed in the lysis solution during the washes and the Buffer F/Fpg incubation.

Alkaline unwinding of all slides was then performed by immersion in an alkaline buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) at 4°C for 40 min. After that, electrophoresis was performed in the same buffer at 1.2 V/cm and 4°C for 20 min. Slides were neutralised with PBS for 10 min at 4°C, washed in distilled water for another 10 min at 4°C and air-dried at room temperature.

DNA in each gel was stained with 1 µg/mL DAPI, and comets were visualised under a fluorescence microscope (NIKON Eclipse 50 i). DNA damage was quantified in 100 randomly selected comets per slide (50 comets in each gel) by measuring the % tail DNA using the image analysis software Comet Assay IV (Perceptive Instruments Ltd). For each slide, the median value of the % tail DNA was calculated. DNA SBs and alkali-labile sites (ALS) are measured in the 'Lysis' slide, while Fpg-sensitive sites were calculated by subtracting the median value of the 'Buffer F' slide from the one obtained in the 'Fpg' slide.

2.4.1. Assay controls

Positive and negative assay controls were included in each electrophoresis run to assess the correct performance of the assay and the inter-assay reproducibility. Positive assay controls

were produced by treating TK6 cells, a human-derived lymphoblastoid cell line, with a) 50 µM MMS for 3 hours to induce oxidised bases or b) 300 µM MMS for 3 hours to induce DNA SBs. Untreated cells were used as negative assay controls. They were prepared, frozen in aliquots and tested (to check if they contained the expected levels of DNA lesions) 1 week before each of the time points (except for the analysis of 1-week frozen samples, in which the assay controls prepared for the analysis of fresh tissues were used). Inclusion of these controls would allow the normalization of the data in case of abnormalities in the results due to technical problems.

2.5. Statistics

Non-parametric U-Mann-Whitney tests were performed to compare the results obtained between fresh and frozen tissues.

3. Results

3.1. Step 1

The results obtained after the different freezing/thawing methods applied to liver tissue samples from untreated animals are shown in Table I. As it can be seen, only approach 1 gave acceptable DNA SBs and Fpg-sensitive sites levels (these results come from several independent analysis). Due to the high levels of DNA SBs obtained following approaches 2, 3, 4 and 5, the results of the comet assay in combination with Fpg could only be obtained in samples processed following approach 1.

Table I. Levels of SBs + ALS and Fpg-sensitive sites obtained after processing liver samples from untreated animals using the different approaches (*i.e.*, freezing and thawing procedures).

Approach	SBs + ALS (% tail DNA)	Fpg-sensitive sites (% tail DNA)
1	4.32 ± 3.58 (n=6)	10.43 ± 2.56 (n=2)
2	~ 90	--
3	~ 90	--
4	82.3	--
5	43.7	--

3.2. Step 2

Fresh and frozen liver, kidney and lung tissue samples from untreated rats or rats administered with 5 or 200 mg MMS/kg b.w., by oral gavage were used. Frozen tissues were snap frozen in

liquid nitrogen and stored at -80°C until comet assay analysis. Samples were processed after 1 week and 1 month, and the thawing process was performed following approach 1.

Assay controls from one experiment in which 1-month frozen samples were analysed, showed a high deviation from the expected % tail DNA. This experiment was discarded due to obvious technical problems related to the performance of the comet assay. Moreover, it was not possible to analyse a slide corresponding to 1-month frozen liver tissue of an animal treated with 200 mg MMS/kg b.w., as it showed a lot of debris.

The levels of DNA SBs + ALS and Fpg-sensitive sites of liver, kidney and lung tissue samples from untreated animals are shown in Figure 2. The levels of DNA SBs were similar in fresh and frozen tissues in all cases. A slight but significant increase in the Fpg-sensitive sites was observed in 1-month frozen kidney when compared with fresh tissues. A similar slight but significant increase was also seen in 1-week but not in 1-month frozen lung tissues. In any case, the observed values can be considered within the normal range for control values.

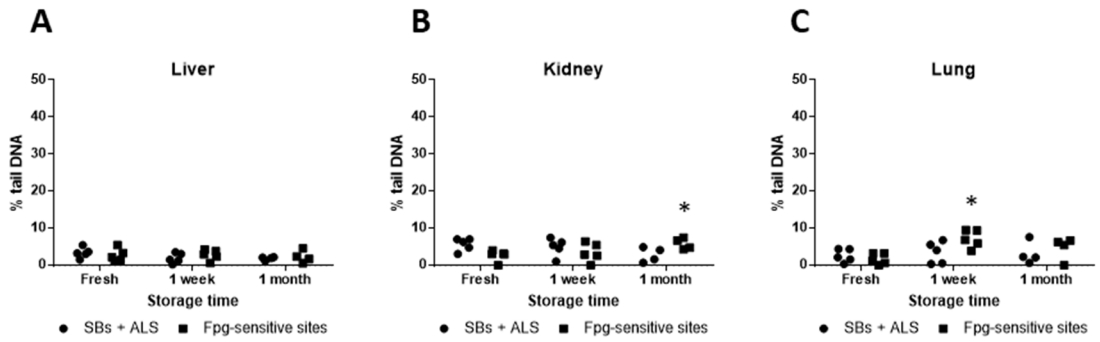

Figure 2. Individual levels of DNA SBs and Fpg-sensitive sites (% tail DNA) found in fresh, 1-week and 1-month frozen (at -80°C) liver (A), kidney (B) and lung (C) tissue samples from untreated animals. *p <0.05 (frozen *vs.* fresh tissue). n=5, except for 1-month frozen tissues (n=4).

In the case of animals treated with 5 mg MMS/kg b.w., a significant increase in the DNA SBs was observed in 1-week frozen lung tissues in comparison with the fresh ones (Figure 3C). Nevertheless, this effect was not observed for 1-month frozen tissues. Frozen liver and kidney showed similar levels of DNA SBs than the fresh ones (Figure 3A and 3B). Regarding Fpg-sensitive sites, a statistically significant decrease was seen in 1-week frozen kidneys compared with the fresh ones, but this difference was not observed after 1 month of freezing (Figure 3B). Frozen liver and lung showed similar levels of Fpg-sensitive sites than the fresh ones (Figures 3A and C).

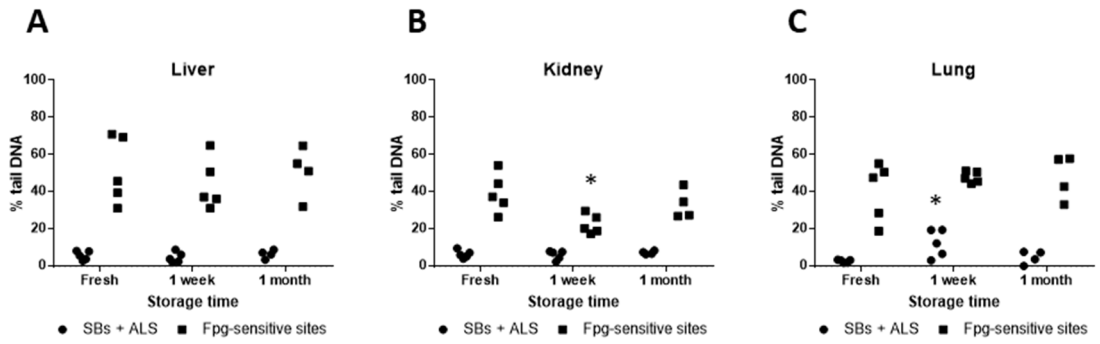

Figure 3. Individual levels of DNA SBs and Fpg-sensitive sites (% tail DNA) found in fresh, 1-week and 1-month frozen (at -80°C) liver (A), kidney (B) and lung (C) tissue samples from rats treated with 5 mg MMS/kg b.w. *p <0.05 (frozen *vs.* fresh tissue). n=5, except for 1-month frozen tissues (n=4).

Finally, no differences in DNA SBs were observed between fresh and frozen tissues of animals administered with 200 mg MMS/kg b.w. (Figure 4).

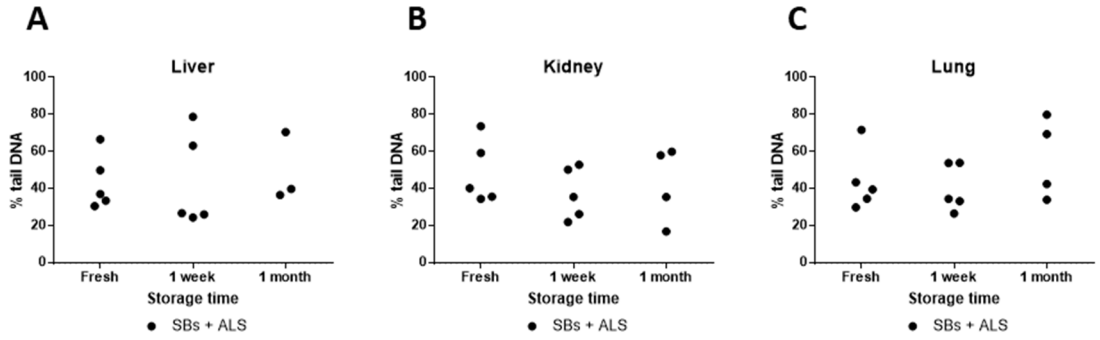

Figure 4. Individual levels of DNA SBs (% tail DNA) found in fresh, 1-week and 1-month frozen (at -80°C) liver (A), kidney (B) and lung (C) tissues from rats treated with 200 mg MMS/kg b.w. *p <0.05 (frozen *vs.* fresh tissue). n=5, except for 1-month frozen liver (n=3), kidney (n=4) and lung (n=4).

Control assay results from the 17 independent experiments which were performed to analyse all the samples are shown in Figure 5. Results are shown by plotting the control assay results from each time point together. As it was mentioned before, one experiment was discarded due to a high deviation from the expected % tail DNA in the assay controls. Untreated assay controls (*i.e.*, untreated TK6 cells) gave the expected results with low variation in both SBs and net Fpg-sensitive sites in all cases (Figure 5A). Positive assay controls for Fpg-sensitive sites (*i.e.*, TK6 cells exposed to 50 µM MMS for 3 hours) and for SBs (*i.e.*, TK6 cells exposed to 300 µM MMS for 3 hours) showed low variation: a slight decrease in the Fpg-sensitive sites (Figure

5B) and a slight increase in the DNA SBs were observed in the assay controls used for the analysis of the 1-month frozen samples (Figures 5B and 5C, respectively).

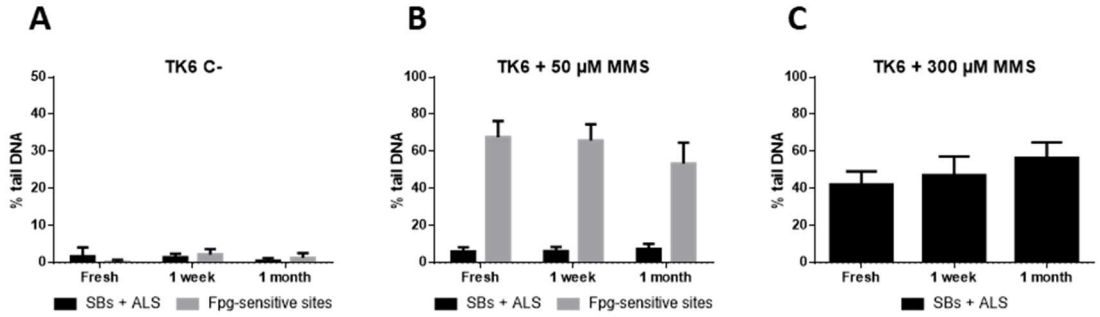

Figure 5. DNA SBs and Fpg-sensitive sites (% tail DNA) of TK6 cells untreated (A), treated with 50 µM MMS for 3 hours to induce oxidised bases (measured as Fpg-sensitive sites) (B) or treated with 300 µM MMS for 3 hours to induce DNA SBs (C). Values are shown as mean ± SD (fresh: n=6; 1 week: n=6 and 1 month: n=5)

In this study, normalization could be performed by correcting the data of an experiment using a correction factor. This correction factor is calculated by dividing the actual assay controls results (*i.e.*, from the experiment that is being normalized) by the expected assay control result. As expected assay controls, the data obtained in previous experiments in which the frozen assay controls are tested (data not shown) or, more commonly, the mean of all the assay control results, can be used. Normalization is done separately for SBs and Fpg-sensitive sites taking into account the correspondent assay control values. This normalization process will be carried out when finishing the analysis of the frozen samples after 3 and 6 months, and 1 year at -80°C. Anyway, according to the variation seen in the assay controls, normalization is not expected to have a big impact in the presented data.

4. Discussion

Taking into account the results obtained when testing the different freezing/thawing procedures, approach 1 was found to be the optimal one for the analysis of frozen liver samples form untreated animals (Figure 1, Table I). It implies snap freezing in liquid nitrogen of a small piece of liver tissue in a cryotube, storage of the sample at -80°C and avoiding tissue thawing until getting a cell suspension in a cold environment on the day of comet analysis. This approach gave acceptable background levels of DNA SBs (% tail DNA = 4.32 ± 3.58, n=6), being within the recommended range for the group mean % tail DNA of liver tissues from vehicle-treated animals (*i.e.*, not exceeding 6% tail DNA; OECD, 2016), and net Fpg-sensitive sites (% tail DNA = 10.43 ± 2.56, n=2) (Table I). However, the rest of the approaches (2, 3, 4 and 5) gave

such high levels of DNA SBs that the Fpg-modified assay could not even be applied (Table I). All these approaches included thawing of the tissue at room temperature, thus indicating that the way the tissue is thawed is crucial. However, since snap freezing of tissues in liquid nitrogen, the most simple and quickest way of freezing samples, gave good results in combination with the thawing procedure described above (*i.e.*, approach 1), the combination of the freezing procedures of approaches 2 to 5 with the successfully thawing procedure from approach 1 was not tested.

The same effect was observed by Jackson and colleagues after testing four different freezing/thawing procedures on the DNA SBs of liver tissues from untreated animals (Jackson *et al.*, 2013). They obtained acceptable background levels when small pieces of tissue were frozen in liquid nitrogen, stored at -80°C and processed in a cold environment preventing the tissue samples to thaw until a cell suspension was obtained (% tail DNA = 6.15 ± 1.62, n=5). However, freezing big pieces of tissue and cutting or crushing them before being processed significantly increased DNA SBs. On the other hand, thawing of the tissues gave very high levels of SBs (% tail DNA = 67.28 ± 20.14, n=5).

As mentioned in the introduction, the comet assay, both in its standard version and in combination with Fpg, has been applied to frozen tissues from different organs in several occasions. Nevertheless, a comparison between the analysis of fresh and frozen tissues has only been done once and regarding DNA SBs in liver and lung from untreated and MMS-treated mice (Jackson *et al.*, 2013). The authors compared DNA SBs in fresh and frozen liver and lung tissues from untreated and MMS-treated mice (25, 75 and 112.5 mg MMS/kg b.w.), but, unfortunately, the time frozen samples were kept at -80°C was not specified. They found a slight but significant increase in frozen lung controls when compared with the fresh ones (though values can be considered within the normal range for untreated animals). Moreover, they showed that leaving the tissues inside a cryotube at room temperature for 15 min before snap freezing in liquid nitrogen did not affect the DNA damage levels substantially (a significant increase in the % tail DNA was only seen in liver and lung samples from untreated animals).

In the present work, for the first time, we have compared the levels of both DNA SBs and net Fpg-sensitive sites in fresh and frozen liver, kidney and lung from untreated and orally MMS-treated rats (5 mg MMS/kg b.w. to induce measurable Fpg-sensitive sites and 200 mg MMS/kg b.w. to induce SBs). The analysis of frozen samples was done after storing the samples for 1 week and 1 month at -80°C. As it is mentioned in the Materials and Methods section, analysis of samples which have been kept frozen for 3 months, 6 months and 1 year is still ongoing;

those results will be included in the manuscript before its submission. Assay controls which consisted in untreated and MMS-treated TK6 cells were included in each experiment to assess the technical variability of assay (inter-experimental variation).

We did not find any increase in the DNA SBs of liver, kidney or lung tissues from untreated animals after 1 week or 1 month at -80°C (Figure 2). Moreover, the group mean % tail DNA is lower than 6% tail DNA for liver tissues in all cases (*i.e.*, fresh, 1-week and 1-month frozen tissues), as recommended by the OECD Guideline for liver tissues of vehicle-treated animals (it does not provide recommendation for other tissues) (OECD, 2016). However, we observed a slight but significant increase on the Fpg-sensitive sites of frozen kidney (1 week) and lung (1 month) tissues (Figure 2B). In all cases, values were within the normal range for control values. Moreover, in the case of the kidney, the increase was seen after 1 week at -80°C but not after 1 month, thus indicating that the observed increase is probably not relevant (Figure 2B). In the case of animals treated with 5 mg MMS/kg b.w., a significant decrease in the Fpg-sensitive sites was observed in 1-week frozen kidney samples in comparison with the fresh ones (Figure 3B). The relevance of this finding is debatable since the level of Fpg-sensitive sites observed in 1-month frozen kidney samples is similar to the levels of the fresh ones (the same phenomenon occurs with the levels of DNA SBs in lung tissue samples). Finally, no statistically significant differences were found in the SBs level between fresh and frozen liver, kidney and lung tissue samples of animals treated with 200 mg MMS/kg b.w. (Figure 4).

Assay controls were included in order to detect technical problems and to assess the comet assay's variation (Figure 5). Moreover, the inclusion of these controls allows data normalization to remove (at least partially) the technical inter-experimental variation. As it was mentioned before, this approach allowed us to discard one of the experiments due to anomalous results in these controls. In all other experiments, negative controls gave the expected results, while both SBs and Fpg-sensitive sites positive controls showed a low-moderate variation. Though normalization of results will be carried out when the analysis of the 3-, 6-months and 1-year frozen samples is completed, up to now it seems that the obtained data will not be substantially affected.

As mentioned in the introduction, although the OECD guideline recognises that tissues or cell nuclei have been successfully frozen for later comet assay analysis, it also requires the demonstration of the laboratory's proficiency in freezing methodologies (OECD, 2016). We have moved a step forward, also applying the Fpg-modified comet assay to 1-week or 1-month frozen tissue samples in order to detect other types of DNA lesions. This approach is not even

covered for fresh tissues in the OECD Guideline, as according to the OECD those necessary protocol modifications still need to be adequately characterised (OECD, 2016).

As it is mentioned in the introduction, some authors freeze tissue samples as a cell suspension using DMSO as a cryoprotectant. Applying the comet assay to frozen cell suspensions led to high % tail DNA values in the liver and duodenum of vehicle-treated male B6C3F1 mice and male Fisher 344/N rats (Recio *et al.*, 2010). Pant *et al.* (2014) found a significant increase in the % tail DNA of frozen cell suspensions, prepared from male liver and male and female kidney of vehicle-treated animals, to levels above their fresh liver historical control ranges, but they do not show their historical control range for kidney. Freezing female liver, and male or female stomach cell suspensions had no effect on the background % tail DNA (Pant *et al.*, 2014). Although cell suspensions were frozen using 10% DMSO in these three studies, it is important to bear in mind that they were also allowed to thaw (at least partially) before slide preparation (Pant *et al.*, 2014; Recio *et al.*, 2010). However, rapid thawing (in a 37°C water bath) and processing of frozen liver cell suspensions of untreated, treated with EMS, 2-acetylaminofluorene (AAF) or cisplatin (CPN) Sprague-Dawley rats (or *in vitro* ɣ-irradiated), gave comparable results to those obtained with fresh preparations (Kraynak *et al.*, 2015). In the same way, Hu *et al.* (2002) showed no significant differences in DNA SB levels measured as tail moment in fresh and 72 h-frozen liver and kidney tissues (-85°C) of untreated and ferric nitriloacetate (Fe/NTA)-treated Sprague-Dawley rats (tissues were digested with collagenase after thawing the samples in a water bath).

Freezing of tissues as cell suspensions may be a good option, or even the only one, depending on the tissue. However, this approach might not be the most optimal in order to overcome the logistical problems due to the handling of a huge number of samples when evaluating multiple tissues from many animals, or when performing the assay in combination with other toxicity assays.

Preparation of specimens in a timely fashion is a critical variable which may affect the results obtained in the comet assay (OECD, 2016), as DNA repair might act as a confounder if samples are maintained fresh (Guerard *et al.*, 2014; Knudsen *et al.*, 2005), or extrinsic DNA damage could be added if it is done under inappropriate conditions (Guerard *et al.*, 2014). In principle, freezing of tissues would allow to maintain a constant and acceptable length of time for the preparation of the specimens, thereby helping to reduce variation due to the processing of several samples.

As it can be concluded from our results, the thawing process seems to be a major risk factor, equally or even more determinant than the freezing process in the preservation of DNA integrity, as it has been shown to dramatically increase the DNA damage detected in liver samples from untreated animals. Thereby, although it is possible to apply the comet assay to frozen tissue samples, extreme caution is needed to avoid unintentional thawing of the samples while processing them. Our results show that the levels of SBs and net Fpg-sensitive sites detected in frozen liver, kidney and lung are comparable to the levels observed in fresh tissues. As it has been mentioned along this document, the stability study of the frozen samples is undergoing.

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Chapter 6

Is oxidative stress involved in the sex-dependent response to OTA renal toxicity?

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Abstract

Ochratoxin A (OTA) is a mycotoxin considered the most powerful renal carcinogen in rodents and classified as a possible human carcinogen. Though its mechanism of action is still unknown, indirect DNA reactivity mediated by oxidative stress has been hypothesised to play an important role. Moreover, large sex-differences have been observed in carcinogenicity studies, being male rats more sensitive than females.

Male and female F344 rats were administered (p.o.) with bicarbonate or 0.5 mg OTA/kg b.w. for 7 days; or with bicarbonate, 0.21 or 0.5 mg OTA/kg b.w. for 21 days. Total glutathione (tGSH) and oxidised glutathione (GSSG) levels, glutathione S-transferase (GST) and superoxide dismutase (SOD) activities were analysed in kidneys. The standard alkaline comet assay was used in combination with Formamidopyrimidine-DNA glycosylase (Fpg) to detect oxidative DNA damage in kidney.

No biologically relevant sex-differences were observed in all the oxidative-stress related parameters analysed. Indeed, no relevant oxidative-stress related response was observed between treated animals and controls. In accordance with the similar OTA levels and histopathological changes between both sexes observed previously in the same animals, and with other oxidative-stress related parameters measured by others, results support that there are no differences between sexes in the oxidative stress response to OTA.

1. Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite produced by several species of Aspergillus and Penicillium. Since this mycotoxin is present in a wide variety of human foodstuff and animal feed (EFSA, 2006; WHO, 2008), human exposure can occur both through consumption of contaminated food commodities or products from animals fed with contaminated feed (NTP, 1989). Thereby, humans are continuously exposed to this mycotoxin (EFSA, 2006; Fink-Gremmels, 2005).

OTA has been proposed as a possible etiological agent of the Balkan Endemic Nephropathy (BEN) and it has also been associated with an increased incidence of urinary tract tumours in humans (Petkova-Bocharova *et al.*, 1988; Pfohl-Leszkowicz *et al.*, 2002; Plestina *et al.*, 1990). However, there is still a lack of epidemiological evidence as other factors or co-factors might be involved in the aetiology of the diseases (Reddy and Bhoola, 2010).

OTA nephrotoxicity has been demonstrated in every laboratory species used, and it is also considered the most powerful renal carcinogen in rats (Lock and Hard, 2004). The available data obtained from different carcinogenicity studies in rodents shows large sex-differences in susceptibility towards OTA-induced tumours: dosing Fischer (F344) rats with OTA for 2 years produced a ten-fold higher incidence of renal tumours in male rats when compared to female rats (Boorman *et al.*, 1992; NTP, 1989), and this sex-biased response has also been observed in other studies using Dark-Agouti and Lewis rats (Castegnaro *et al.*, 1998; Son *et al.*, 2003). Other studies have deepened into the effect of sex on OTA toxicity *in vivo* from the toxicokinetic point of view (Vettorazzi *et al.*, 2011, 2010, 2009; Zepnik *et al.*, 2003).

Unfortunately, the exact mechanism of action by which OTA induces tumours is still unknown and several hypotheses have been proposed to contribute, totally or partially, to it (WHO, 2008). Among them, indirect DNA reactivity mediated by oxidative stress has been supported by several authors. Several studies have demonstrated that OTA inhibits the nuclear factor, erythroid 2-like 2 (Nrf2) oxidative stress response pathway, which would affect glutathione synthesis and recycling, oxidoreductases activity, and phase II metabolism inducibility, thus rendering the tissue more vulnerable to oxidative stress (Limonciel and Jennings, 2014). A downregulation of genes under transcriptional control of Nrf2 was also observed in the kidney of rats fed OTA up to 12 months (Marin-Kuan *et al.*, 2006). The effects observed at mRNA level were later confirmed as biologically relevant as OTA also decreased the protein expression of several markers of the Nrf2-regulated gene battery in kidney *in vivo*, which resulted in

oxidative DNA damage, and *in vitro* in the NRK renal cell line and in primary hepatocyte cultures (Cavin *et al.*, 2007). Arbillaga *et al.* (2007a), found that several genes implicated in the oxidative stress response were up-regulated in the human renal cell line (HK-2) following OTA exposure during 6 and 24 h, but identified down-regulation as the predominant effect in a repeated-dose study carried out in F344 rats (Arbillaga *et al.*, 2008). A differential expression of genes involved in the response to oxidative stress was also seen *in vivo* and *in vitro* by Lühe *et al.* (2003). In the same line, using both the Eker rat model of increased susceptibility to renal tumour formation and the corresponding wild-type strain, Stemmer *et al.* (2007) found that OTA treatment down-regulated the expression of several phase I and phase II enzymes in both strains and deregulated the expression of several genes involved in the response to DNA damage (including oxidative stress) in Eker rats.

On the other hand, other effects related to oxidative stress production have been observed after OTA exposure. Omar *et al.* (1990) came to the conclusion that OTA stimulates lipid peroxidation by complexing Fe3+, which may facilitate its reduction, although the specific specie responsible for initiating lipid peroxidation was not identified. It has been established that OTA leads to lipid peroxidation both *in vitro* (Klarić *et al.*, 2007) and *in vivo* (Abdel-Wahhab *et al.*, 2005; Ferrante *et al.*, 2006; Ozçelik *et al.*, 2004), to a decrease of glutathione (GSH) levels *in vitro* (Klarić *et al.*, 2007; Schaaf *et al.*, 2002) and *in vivo* (Meki and Hussein, 2001), and to an increase of the kidney's protein carbonyl levels after 21 days of OTA-treatment (Domijan *et al.*, 2005). It has also been observed that OTA causes a dose-dependent increase of reactive oxygen species (ROS) (Baldi *et al.*, 2004) as well as oxidative DNA damage *in vitro* (Arbillaga *et al.*, 2007b; Schaaf *et al.*, 2002), and it is considered to significantly increase oxidative DNA damage *in vivo* (Kamp *et al.*, 2005; Mally *et al.*, 2005). OTA exposure to HepG2 cells decreased the intracellular zinc concentration (considered a potential antioxidant), induced ROS production, 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation and decreased superoxide dismutase (SOD) activity (Zheng *et al.*, 2013). In fact, treatment of OTA-pretreated Wistar rats with SOD and catalase prevented enzymuria, proteinuria, creatinemia and increased urinary excretion of OTA (Baudrimont *et al.*, 1994).

Thus, it appears clear that oxidative stress might be implicated in OTA toxic response. However, the influence of sex in OTA-mediated kidney oxidative stress response has not been specifically studied. Indeed, this might be an important aspect to evaluate as sex differences regarding oxidative stress response have been observed in other scenarios such as after giving a high cholesterol diet to Wistar albino rats (Al-Rejaie *et al.*, 2012), acetaminophen to CD-1 mouse (Hoivik *et al.*, 1995) or cisplatin to Swiss albino mice (Naseem *et al.*, 2015). Besides,

Lash *et al.* (1998) found that the rate of *S*-(1,2,2-trichlorovinyl) glutathione (TCVG) formation in isolated kidney cells from male and female F344 rats were similar, but kidney cytosol and microsomes from males exhibited higher amounts of TCVG formation than the corresponding fractions from females, for both F344 rats and B6C3F1 mice. Regarding *in vitro* studies, vascular smooth muscle cells (VSMC) isolated from male rat aorta were found to be much more susceptible to radiation-induced stress (measured by ROS production) than the female ones (Malorni *et al.*, 2008).

Several studies have measured kidney oxidative stress status using different rat species and with different dosages after OTA administration. However, all of them have used male rats. To the authors knowledge, only one study (Hibi *et al.*, 2011) analysed oxidative DNA damage (8-hydroxy-2'-deoxyguanosine levels) in both sexes after 4 and 13 weeks of OTA administration in diet (approximately 0.4 mg/kg b.w.). No differences between sexes were found in oxidative DNA damage or at histopathological level. This in agreement with our recently published study carried out in male and female F344 rats (Pastor *et al.*, 2018), where slightly higher signs of toxicity were found in kidney histopathology in males after 7 days of 0.5 mg OTA/kg b.w. daily administration but no differences were found after 21 days of treatment.

Due to the different tumour incidence found between both sexes (NTP, 1989) and the evidences suggesting an OTA indirect mechanism of action through oxidative stress, the present study aims at measuring different oxidative stress-related parameters such as glutathione-S-transferase (GST) activity, total (tGSH) and oxidised (GSSG) glutathione levels and superoxide dismutase activity (SOD) in kidney of both male and female F344 rats. Moreover, in order to relate these endpoints with oxidative DNA damage, the comet assay in combination with Formamidopyrimidine-DNA glycosylase (Fpg) was carried out in kidney tissue.

2. Materials and methods

2.1. Chemicals and reagents

Commercial available kits were used for the determination of total (cytosolic and microsomal) GST activity (Glutathione S-Transferase Assay Kit®, Item No. 703302, Cayman Chemical), tGSH and GSSG glutathione (Glutathione Assay Kit®, Item No. 703002, Cayman Chemical), and SOD activity (SOD determination kit®, Item No. 19160, Sigma-Aldrich). For protein quantification, Protein Assay Dye Reagent Concentrate (Item No. 500-0006, Bio-Rad) and Standard Bovine Serum Albumin (BSA, Item No. A3803, Sigma) were used. The SOD standard from bovine

erythrocytes (Item No. S2515), 2-vinylpyridine (Item No. 132292) and EDTA were purchased from Sigma-Aldrich. Dulbecco's Phosphate Buffered Saline (PBS) without Ca^{+2} and Mg^{+2} 10x from Lonza (Item No.BE17-515Q) was used to prepare PBS 1x washing solutions for tissues or comet assay slides. Saline for washing tissues for SOD activity determination was purchased from Grifols (Item No. 825083). The salts KH_2PO_4 and $K_2HPO_4 \cdot 3\ H_2O$ used to prepare buffer A and B for tissue preparations were obtained from Panreac AppliChem and Merck KGaA, respectively. For sucrose buffer (pH 7.4), 0.25 M sucrose, 10 mM Tris and 1 mM EDTA were purchased from Sigma.

For the comet assay, low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, BSA, methyl methanesulfonate (MMS) and 4',6-diamidino-2-phenylindole (DAPI) (Item No. D9542, Sigma) were purchased from Sigma-Aldrich. NaCl, NaOH, Na_2HPO_4 and KCl were purchased from PanReac AppliChem and Dulbecco's Phosphate Buffered Saline (DPBS) 1x (Ref. 14190-094) for mixing cell suspensions with agarose was purchased from Gibco. Fpg was a gift from Prof. Andrew Collins (University of Oslo). Ro 19-8022 (Ro), which specifically produces oxidised purines (mainly 8-oxo-7,8-dihydroguanine; 8-oxoGua) in the presence of visible light, was kindly given by Hoffmann-La Roche.

2.2. Samples

This study was carried out using samples obtained in a previous study (Pastor *et al.*, 2018). The study was approved by the Ethics Committee on Animal Experimentation of the University of Navarra. Briefly, kidneys were obtained from male and female Fischer 344 (F344/IcoCrl) rats gavaged daily for 7 days with OTA (0.50 mg/kg b.w.) or vehicle ($NaHCO_3$) (n= 6 per treatment group and sex) or for 21 days with OTA (0.21 mg/kg b.w. or 0.50 mg/kg b.w.) or vehicle ($NaHCO_3$) (n= 6 per treatment group and sex). Unfortunately, some rats were euthanised for ethical reasons while conducting the study. For that reason, for the different determinations of the 21-days study, 4 samples of male control group and 5 samples of female group treated with 0.21 mg/kg b.w. OTA were available.

Twenty-four hours after the last administration, animals were sacrificed by decapitation and their organs were removed. Left kidneys were longitudinally cut in two halves. One half was excised in pieces of approximately 50-100 mg, containing both cortex and medulla. The pieces for the subsequent determination of glutathione (tGSH and GSSG) content and GST activity were washed in ice-cold PBS (phosphate buffered saline), pH 7.4, and the pieces for the subsequent determination of SOD activity were washed with ice-cold saline (0.9% NaCl), to

remove any red blood cells and clots. All the pieces were then introduced in labelled cryotubes, snap frozen in liquid nitrogen and stored at -80°C until analysis.

For the comet assay, approximately 2 x 3 x 5 mm kidney samples, containing both cortex and medulla, were cut, placed in labelled cryotubes and snap frozen in liquid nitrogen. Samples were also stored at - 80°C until the comet assay was performed.

2.3. Determination of total GST activity

Total GST activity (cytosolic and microsomal) was spectrophotometrically determined at 340 nm by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione using a commercial available kit from Cayman Chemical. One unit of enzyme is defined as the amount of enzyme able to conjugate 1.0 nmol of CDNB with reduced glutathione per minute at 25°C. The assays were performed by using the provided solutions and following the kit-included protocol.

For that purpose, frozen kidney samples were homogenised in 7.5 mL of ice-cold buffer A (100 mM phosphate, pH 7.0, containing 2 mM EDTA) per gram of tissue, using a Teflon pestle homogenizer at 600 rpm. The homogenate was centrifuged at 8000 x g for 15 min at 4°C, and the protein concentration of the supernatants was assayed using the Bradford assay by extrapolating from known concentrations of standard BSA. For the assay, 6 mg/mL (120 µg) of protein were used.

2.4. Quantification of tGSH and GSSG levels

Total glutathione (tGSH) (both reduced and oxidised) concentration was spectrophotometrically assessed at 405 nm by a recycling method in which reduced glutathione (GSH) reacts with DTNB (5,5'-dithio-bis-2-[nitrobenzoic acid], Ellman's reagent) to produce a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) using a commercial available kit from Cayman Chemical. Since glutathione reductase is used in the kit, both GSH and GSSG glutathione are measured and the assay reflects tGSH. For exclusive determination of GSSG the assay was accomplished by first derivatizing with 2-vinylpyridine following the kit instructions.

For that purpose, frozen kidney samples were homogenised in 5 mL of ice-cold buffer B (50 mM phosphate, pH 6-7, containing 1 mM EDTA) per gram of tissue, using a Teflon pestle homogenizer at 600 rpm. The homogenate was centrifuged for 15 min at 8000 x g and 4°C, and 50 µL of supernatant was used for the determination. Thereafter the protein concentration of

the supernatants was assayed using the Bradford assay by extrapolating from known concentrations of standard BSA. Following the kit instructions, supernatants were deproteinated before assaying tGSH and GSSG levels.

Quantification of tGSH or GSSG was achieved following the End Point Method calculations indicated in the kit and, and results were expressed in nmol/mg protein.

2.5. Determination of SOD activity

SOD activity was determined spectrophotometrically by an indirect method using a commercially available kit from Sigma. The measurement method is based on the principle that xanthine reacts with xanthine oxidase to generate superoxide radicals, which react with a highly water-soluble tetrazolium salt (WST-1) to produce a water-soluble formazan dye (WST-1 formazan). SOD activity is then determined spectrophotometrically at 440 nm by measuring the degree of inhibition of this reaction. The assays were performed by using the provided solutions and following the kit-included protocol.

Frozen kidney samples were homogenised in 900 µL of ice-cold sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4) per 100 mg of tissue, using a Teflon pestle homogenizer at 600 rpm. The homogenate was centrifuged for 15 min at 8000 x g and 4°C, and the protein concentration of the supernatants was assayed using the Bradford assay by extrapolating from known concentrations of standard BSA. For the assay, 0.1 mg/mL (2 µg) of protein were used.

2.6. Comet assay

Tissue samples were processed as described by Jackson *et al.* (2013). The cryotubes containing the tissues were placed on dry ice and one sample was processed at a time until cells were embedded in agarose. A drop of Merchant´s buffer (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) was placed on the tissue to create a protective icecap. The tissue was then transferred into a cylindrical sieve (*i.e.*, a stainless-steel cylindrical tube of 5 mm diameter with a stainless-steel screen of 0.4 mm fitted inside) previously immersed in 3 mL ice-cold Merchant's buffer using cold tweezers. Tissues were homogenised by pressing them through the sieves moving a plastic plunger up and down several times. Kidney cell suspensions were centrifuged for 5 min at 390 x g and 4°C and the pellet was resuspended in 3 mL of ice-cold Merchant´s buffer.

Thirty microliters of the cellular suspension were mixed with 140 µL of 1% low melting point agarose in PBS at 37°C. Immediately, two drops of 70 µL each were placed on a glass

microscope slide (pre-coated with 1% normal melting point agarose in distilled water and dried) and covered with 20 x 20 mm coverslips. Gels were set on a metal plate on ice and the coverslips were removed. Overnight lysis at 4°C was performed by immersing the slides in lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-base, pH 10.0, 1% Triton X-100 prior to use).

Three slides were prepared from each cell suspension: the 'Lysis' slide, the 'Fpg' slide and the 'Buffer F' slide. After lysis, the 'Fpg' and 'Buffer F' slides were washed three times (5 min each) with the enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/mL BSA, pH 8.0-Buffer F) at 4°C. A drop of 45 µL of Buffer F or Fpg was added on top of the corresponding gels, covered with a 22 x 22 mm coverslip and incubated in a humidified atmosphere at 37°C for 30 min. Meanwhile, the 'Lysis' slides were kept immersed in the lysis solution.

Alkaline DNA unwinding was performed by immersing the slides in an alkaline buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) at 4°C for 40 min. After that, 20 min electrophoresis was performed in the same buffer at 1.2 V/cm and 4°C. Following electrophoresis, slides were washed with PBS for 10 min at 4°C, with distilled water for another 10 min at 4°C and air-dried at room temperature.

Afterwards, DNA in each gel was stained with 30 µL of 1 µg/mL DAPI, and comets were visualised under a fluorescence microscope (NIKON Eclipse 50 i). DNA damage was quantified in 100 randomly selected comets per slide (50 comets in each gel) by measuring the % tail DNA using the image analysis software Comet Assay IV (Perceptive Instruments Ltd). For each slide, the median value of the % tail DNA was calculated. DNA strand breaks (SBs) and alkali-labile sites (ALS) are measured in the 'Lysis' slide, while Fpg-sensitive sites (*i.e.*, an indicator of oxidative DNA damage) were calculated by subtracting the median value of the 'Buffer F' slide from the one obtained in the 'Fpg' slide for each tissue sample. For the final results, the mean of the median obtained per animal was calculated.

Positive and negative assay controls were included in each electrophoresis run to assess a correct performance of the assay and the inter-assay reproducibility. Positive assay controls were produced by treating V-79 cells, derived from Chinese hamster lung fibroblasts, with a) 1 µM Ro 19-8022 plus light to induce oxidised bases or b) 200 µM MMS to induce DNA SBs. Untreated V-79 cells were used as negative assay controls. These controls were prepared and frozen in aliquots.

Moreover, a sample of each of the treatments from both sexes was included in each electrophoresis run to minimize the potential influence of the inter-experimental variation in the results.

2.7. Statistics

Non-parametric one-way analysis of variance (*i.e.*, Kruskal-Wallis test), followed by Mann-Whitney multiple-comparison U-tests, were performed to compare the results obtained between different doses within the same sex and also to compare between both sexes treated with the same dose.

3. Results

3.1. GST activity

No statistically significant differences were found between control and OTA-treated groups or both sexes, neither after 7 days nor after 21 days of treatment (Figures 1A and 1B). However, GST activity tended to be reduced in the female groups treated with 0.21 or 0.5 mg OTA/kg b.w. after 21 days of treatment when compared to the control group (Figure 1B).

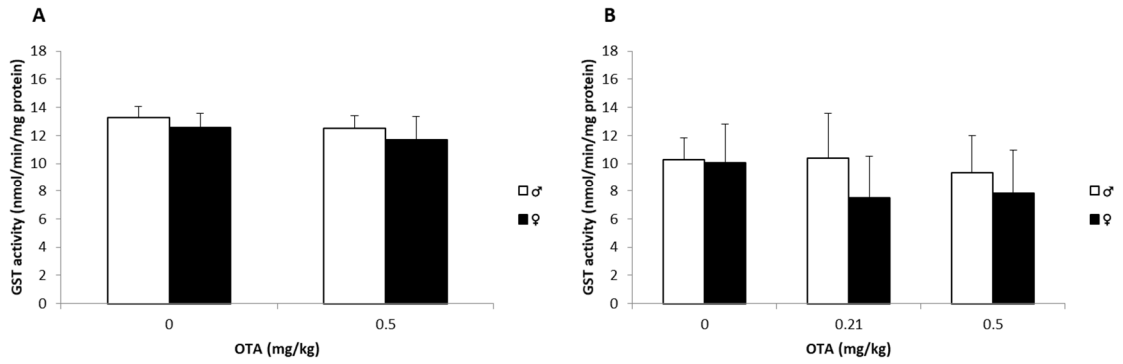

Figure 1. GST activity measured in kidney tissue of male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days (A) or with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days (B). Values are shown as average ± SD.

3.2. tGSH and GSSG levels

In the 7-days study, tGSH levels in the kidney were higher in female than in male rats, although this difference was only statistically significant between control groups ($p=0.021$ between control groups, and $p=0.078$ between OTA-treated groups). OTA-treatment did not alter tGSH levels neither in males nor in females (Figure 2A).

Again, in the case of control animals from the 21-days study, females presented statistically significant higher levels of tGSH than males (p=0.032). OTA treatment diminished the basal sex-differences as a slight dose-dependent increase was observed in males, while in females tGSH levels tended to decrease with the treatment. However, no statistically significant differences were observed between treated and control groups for both sexes (Figure 2B).

Regarding GSSG levels, both control and OTA-treated females showed higher levels than males at 7 days (Figure 2C). This difference was not observed at 21 days (Figure 2D). OTA treatment did not affect GSSG levels in both sexes neither at 7 days nor at 21 days.

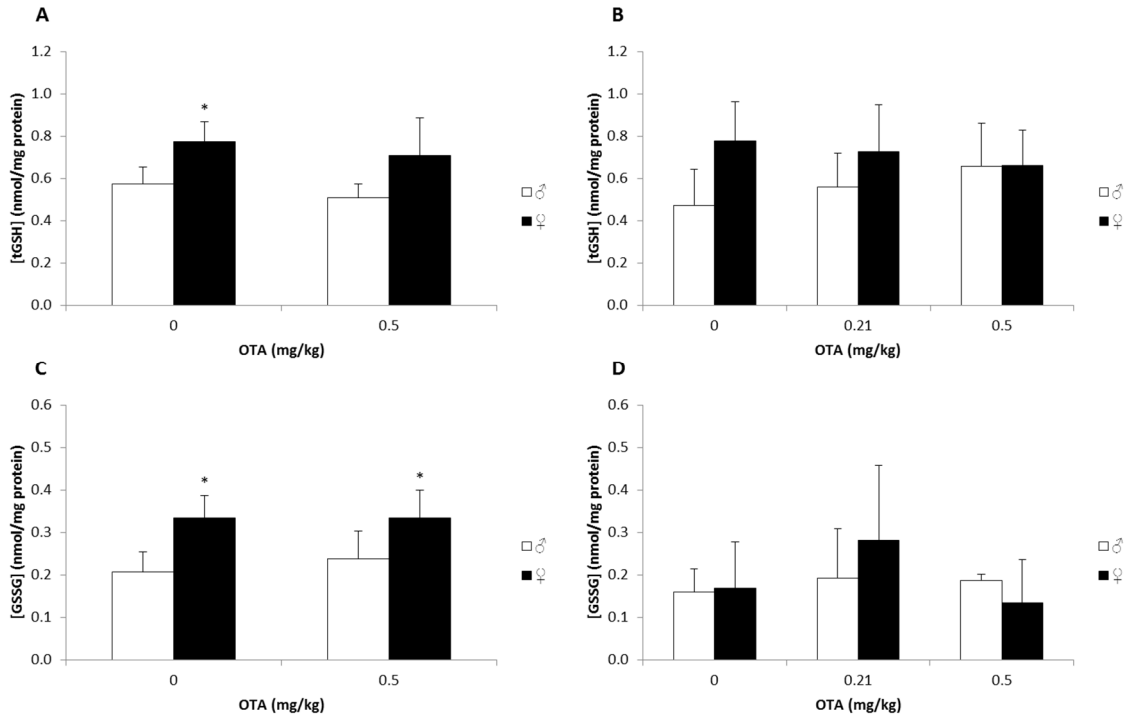

Figure 2. tGSH content measured in kidney tissue of male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days (A) or treated with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days (B). GSSG content measured in kidney tissue of male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days (C) or treated with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days (D). Values are shown as average ± SD. *Statistical significance ($p < 0.05$) females *vs.* males.

3.3. SOD activity

OTA treatment for 7 or 21 days did not alter significantly kidney SOD activity levels neither in males nor in females. Besides, there were not any statistically significant differences in kidney SOD activity levels between males and females (Figure 3).

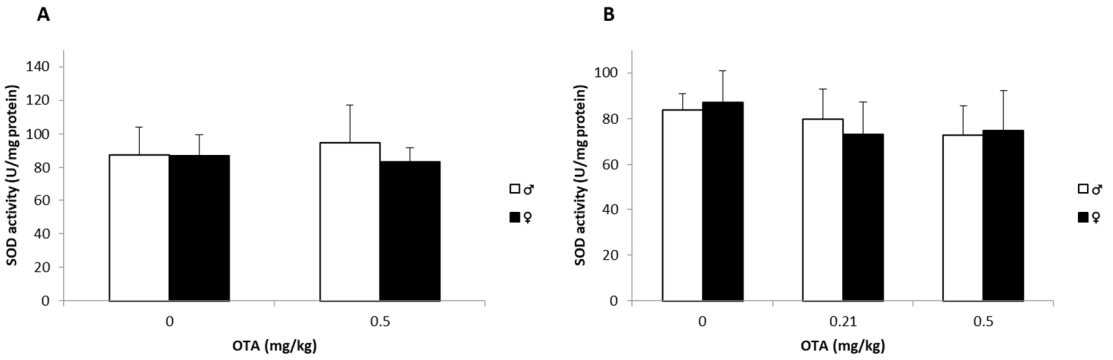

Figure 3. SOD activity measured in kidney tissue of male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days (A) or treated with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days (B). Values are shown as average ± SD.

3.4. Comet assay

OTA treatment for 7 or 21 days did not induce a significant increase of the DNA damage, neither as DNA SBs (plus ALS) nor as oxidised bases (*i.e.*, Fpg-sensitive sites), in kidney tissue from male and female rats (Figures 4A and 4B). Differences between both sexes were also not observed.

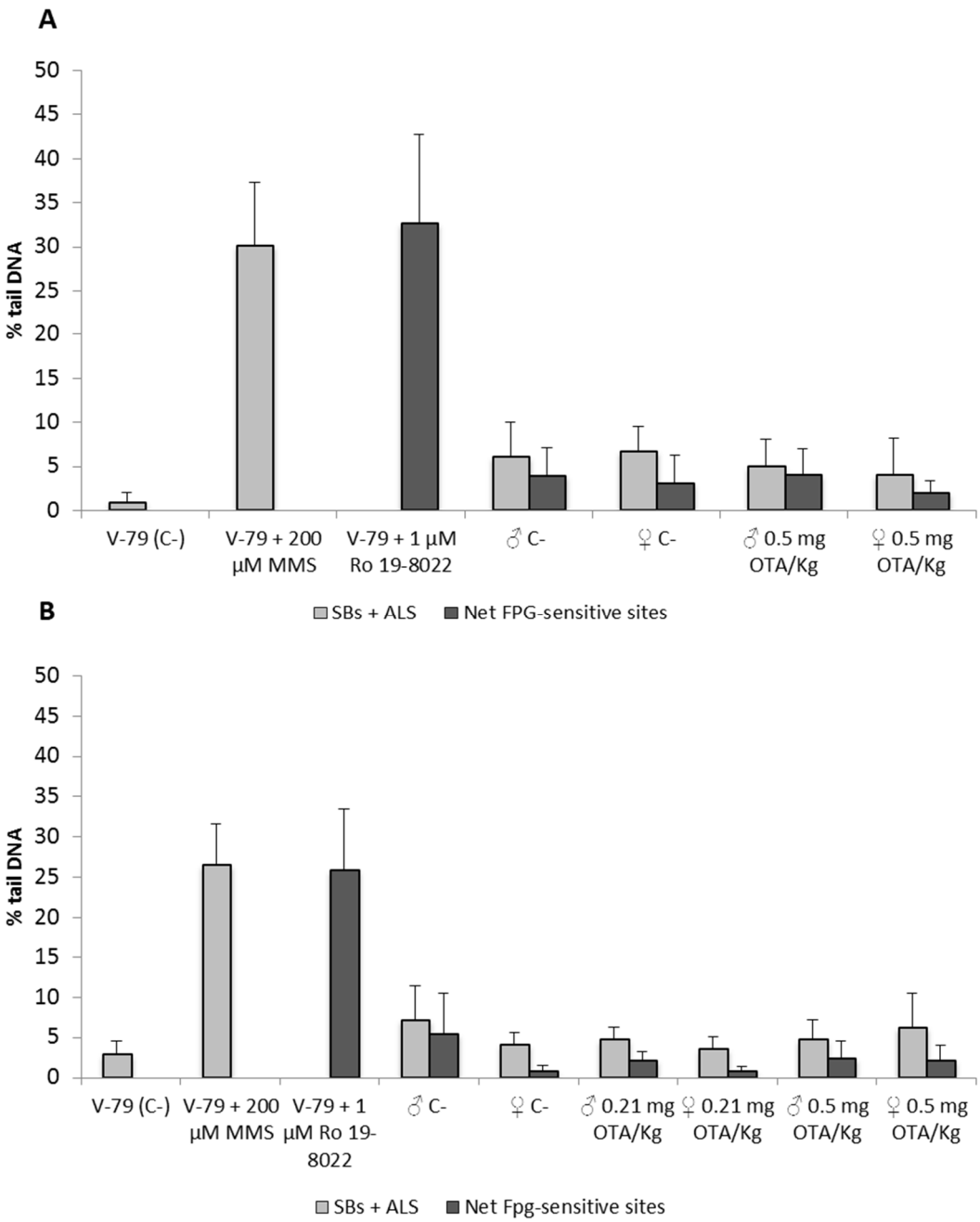

Figure 4. DNA SBs (plus ALS) and Fpg-sensitive sites measured in kidney tissue of male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days (A) or with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days (B). Results from assay controls with V-79 are also shown. Values are shown as mean ± SD.

4. Discussion

Low levels of chronic oxidative stress have been associated with carcinogenesis (Klaunig and Kamendulis, 2004). A great deal of research carried out until today supports that OTA

mechanism of action is related with a certain degree of oxidative stress (reviewed by Marin-Kuan *et al.*, 2011). Even if some hypotheses have been proposed (Cavin *et al.*, 2007; Limonciel and Jennings, 2014), the exact role of oxidative stress in OTA-induced renal carcinogenesis remains unknown.

What seems to be clear is that OTA induced a higher incidence of kidney tumours in male rats than in females (Castegnaro *et al.*, 1998; NTP, 1989; Son *et al.*, 2003). Thus, the aim of the present study was to evaluate the antioxidant response of both males and females after exposure to OTA doses (0.21 and 0.5 mg/kg b.w.) known to produce renal tumours in 2 years-bioassays (Castegnaro *et al.*, 1998; NTP, 1989). For that purpose, kidney samples from F344 rats treated for 7 and 21 days with OTA by gavage (from Pastor *et al.*, 2018) were analysed for GSH levels, GST and SOD activity and DNA damage evaluated by the comet assay. In Pastor *et al.* (2018) and, more concretely, in the same samples evaluated in the present study, several sex-differences related with kidney transporters regulation were observed. However, OTA concentration in plasma and kidneys was similar in both sexes. Histopathology revealed that, after 7 days of treatment with 0.50 mg OTA/kg b.w., the number of animals or the intensity of glomerulonephritis, tubulonephrosis or alterations in the collecting ducts was slightly higher in males than in females. However, after 21 days, even if the incidence or the severity of the lesions increased in both sexes, sex differences disappeared for the two doses evaluated. In the present study, no biologically relevant sex-differences were observed in all the oxidative-stress related parameters analysed: total GST activity, tGSH and GSSG levels, SOD activity and oxidative DNA damage. Indeed, no relevant oxidative stress related response was observed between treated animals and controls. These results are in agreement with an *in vivo* study carried out with OTA in male and female rats (Hibi *et al.*, 2011), where no differences related with oxidative stress damage (measured as nuclear 8-OHdG) were observed neither between rats fed 5 ppm OTA for 4 weeks and controls nor between both sexes. Equally, no differences at histopathological level were observed.

In the present study, GST activity was evaluated as it is a well-known phase II-metabolism enzyme that plays a key role in cellular detoxification. It conjugates xenobiotics to glutathione, thereby neutralizing their electrophilic sites, and rendering the products more water-soluble in order to excrete them. This enzyme, together with GSH, might also play a role in the metabolic fate of OTA. Indeed, some authors considered that a small portion of OTA bioactivates into hydroquinone (OTHQ) and quinone (OTQ) derivatives, that are in turn further metabolised into glutathione conjugates (OTHQ-GSH) (Dai *et al.*, 2002). This reaction has been proposed to be also catalysed by GSTs by some authors (Pfohl-Leszkowicz and Castegnaro, 2005). Some

authors have proposed that ROS could be produced during this reaction (Gillman *et al.*, 1999). Unfortunately, even though certain GST polymorphisms have been associated with an increased risk of Balkan Endemic Nephropathy (Reljic *et al.*, 2014), a human disease that has been partially related to OTA exposure, and with an increased DNA damage (Lebrun *et al.*, 2006), the role of GST has not been deeply explored in relation to OTA mechanism of action. In the present study, no statistically significant sex-differences in GST activity have been observed. However, a tendency to a dose-dependent decrease of GST activity was observed mainly in females treated with OTA after 21 days. In the hypothetic situation that GST activates OTA to more DNA-reactive quinone derivatives, our result would support a slight tendency to lower production of these metabolites in females (less sensitive to tumour formation than males). Indeed, Tozlovanu *et al.* (2012) found that Dark-Agouti male rats generate higher levels of GSH-OTA conjugates than females. But, due to the fact that GST might also reduce free radicals, males could be more effective fighting against oxidative stress. Due to the conflicting results on the role of GSH in OTA toxicity (reviewed by Turesky, 2005) its levels were also measured in the present study in both sexes. Slightly higher levels of tGSH were detected in control females than in males. Even if not statistically significant, a slight dose-dependent increase of tGSH was observed in OTA-treated males, while females tended to show a dose-dependent decrease. In general, the sex-differences observed in the present study are too slight to consider them as biologically relevant and might be within the normal physiological levels. In addition, no differences regarding SOD activity were observed between control and OTA treated animals, as well as between sexes. SOD activity was evaluated as it is an important enzyme involved in oxidative stress detoxification that has been shown to be related to OTA nephrotoxicity (Baudrimont *et al.*, 1994; Ciarcia *et al.*, 2016).

Our results are in accordance with several *in vivo* studies carried out with OTA in males. Chong and Rahimtula (1992) concluded that there were no significant changes in GST and SOD activities in kidney cortex supernatants of Sprague-Dawley rats treated daily for 4 days with 0.5, 1 or 2 mg OTA/kg b.w., nor in cytosol isolated from rats 10 min to 6 h after a single dose of 10 mg OTA/kg b.w. Accordingly, another acute oral treatment of male F344 rats (up to 2 mg OTA/kg b.w., 24 h) (Gautier *et al.*, 2001) did not induce an increase in the lipid peroxidation marker malondialdehyde in rat plasma, kidney and liver, nor in the DNA damage marker 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in kidney. Žlender *et al.* (2009) also did not find differences in GSH (and 8-OHdG levels) in kidneys from OTA-treated rats by gavage at different doses for 10 days. Moreover, more recently Taniai *et al.* (2014) and Qi *et al.* (2014), using similar experimental designs to ours (F344 male rats, 0.21 mg OTA/kg b.w. in $NaHCO_3$ for 28

days) did not find significant differences in tGSH nor in GSSG levels between OTA-treated and control animals. Moreover, at this timepoint, Qi *et al.* (2014) did not find differences in SOD activity nor in other oxidative stress related parameters (*i.e.*, ROS, malondialdehyde or 8-OHdG) as well as DNA SBs in the comet assay.

In contradiction to our results interpretation, Palabiyik *et al.* (2013) found a significant decrease of GSH levels and an increase of SOD activity in male Sprague-Dawley rats treated with 0.5 mg OTA/kg b.w. (dissolved in corn oil and 10% DMSO) for 14 days. On the other hand, using the same rat strain but administering 3 mg OTA/kg b.w. in the diet for 15 days, Abdel-Wahhab *et al.* (2005) considered that OTA significantly reduced SOD activity. Meki and Hussein (2001) also found a reduction of GSH levels and SOD and GST activities in OTA treated Sprague-Dawley rats (0.25 mg OTA/kg b.w. in $NaHCO_3$ by oral gavage for 4 weeks) compared to controls. It should be noted that, when comparing all these different studies (in accordance or discordance to ours), not only the differences expected from different *in vivo* experimental designs or rat strains should be taken into account. Indeed, the major factors explaining contradictory results might be related with tissue preparation (freezing or not before homogenization), extract preparation (different speed and time of centrifugation or extraction buffers may lead to different cytosolic extracts), the amount of tissue or protein loaded, and the methods and calculations used for the different determinations. Indeed, these technical differences make comparisons among studies almost impossible. On the other hand, interpretation of results is generally based on statistical significance, without taking into account if the differences obtained are within the normal physiological variability of these enzymes or the protein levels in the rat tissue, which unfortunately has not been studied. For example, Ozçelik *et al.* (2004) reported a decrease in SOD activity in OTA-treated Wistar rats (289 µg OTA /kg b.w. in drinking water for 4 weeks) *versus* control animals. However, even that the differences were statistically significant (0.94 ± 0.14 *vs.* 0.78 ± 0.13 U/mg protein), both results might be within the normal physiological variability for SOD activity. Similarly, in the study from Bertelli *et al.* (2005), in which similar kits to the ones used in the present study for tGSH levels and SOD activity determination were used, the authors concluded that both the GSH/GSSG ratio and SOD activity were reduced by OTA treatment in Wistar rats (289 µg OTA/kg b.w. by oral gavage for 14 days). However, the mean values for SOD activity were 41 and 51 U/mg of protein for the OTA-treated and control groups, respectively. Taking into account that the standard deviation for this study was around 10, it seems very likely that both results are within the normal physiological ranges.

A similar situation can be found when comparing DNA SBs or oxidised bases detected with the comet assay. In accordance with the rest of the oxidative stress-related parameters measured, no differences due to OTA treatment or sex could be observed in our study. In agreement with our results and under similar experimental *in vivo* exposure conditions, Kamp *et al.* (2005) and Qi *et al.* (2014) did not find a significant increase on the DNA detected with the standard alkaline comet assay in kidney tissue of F344 rats. However, Kamp *et al.* (2005) concluded that OTA mediated-oxidative stress was detected when the enzyme Fpg was used in combination with the comet assay. Having a close look to the published data, the oxidised bases detected increased from 0.7% (approx.) in the control group to 3% (approx.) in the highest dose group (very low values even for tissues from non-treated animals); the statistically significant difference must be carefully interpreted in this case. Moreover, there was not a dose-dependent relationship. The same interpretation would apply for the increased DNA SBs and oxidised bases observed in Domijan *et al.* (2006), in which kidneys from Wistar rats orally administered with 5 ng/kg, 0.05 and 5 mg/kg for 15 days were used. In this study, though the DNA SBs and oxidised base levels were very low in all groups (*i.e.*, DNA SBs increased from 0.4% tail DNA in the vehicle-treated group to 5.6% in the highest-dose group, while oxidised bases increased from 0.7 to 7.2 % tail DNA), they found a dose-response relationship. Kuroda *et al.* (2014) performed the comet assay in renal outer medulla of gpt delta rats treated with 0, 70, 210 and 630 µg/kg OTA by oral gavage for 4 weeks. They also found a small but significant increase in the level of DNA SBs in treated animal (*i.e.*, from 4% DNA in tail in control group to about 10-12% in treated animals); however, the increase was not dose dependent. Aydin *et al.* (2013) found a significant increase of DNA SBs in Sprague-Dawley rats treated p.o. with 0.5 mg OTA/kg b.w. for 14-15 days (*i.e.*, from about 11% DNA in tail in control group to about 21% in treated animals).

In another study with a similar experimental design than ours (Mally *et al.*, 2005), the author concluded that a small but significant increase in DNA breakage was observed in the kidney, while when using Fpg, a significant increase over control was only evident in the high-dose (2000 µg OTA/kg b.w.) group. Unfortunately, the authors reported the results as tail moment, a measure that hinders the interpretation of the basal DNA damage in control animals, a good indicator of quality performance in the comet assay. Indeed, by comparing the comet images shown in their study and the given tail moment values, negative control animals seem to show very high % tail DNA (about 50%) when using Fpg. Moreover, it is not clear if DNA SBs were substracted in the calculations in order to get net Fpg-sensitive sites (*i.e.*, oxidised bases level). All these issues, together with the fact that the comparable dose to our study (500 µg OTA/kg

b.w.) was not reported in the results of the comet assay in presence of Fpg, make it impossible to correctly compare the results with the present study. Similarly, in Želježić *et al.* (2006), DNA damage is expressed by a formula (DNA damage potency) that makes difficult the comparison with other studies and to really know the basal DNA damage of control animals.

Overall, studies measuring oxidative stress related parameters after OTA treatment *in vivo* show controversial results. The main differences, apart from differences in the experimental design, might be due to i) different technical protocols and performance, ii) differences in calculations and reporting of results (formulas, etc) and iii) differences in the interpretation of the results (statistical differences *vs.* physiologically relevant differences).

Moreover, even if OTA has produced positive responses in oxidative stress related parameters in many *in vitro* studies, the situation is not so crystal clear *in vivo*. As already discussed by Gautier *et al.* (2001), the discrepancy between the generation of ROS *in vitro* and *in vivo* and the resultant oxidative damage may be explained by the protective action of the antioxidant defences present in the animal model. Indeed, this was further supported in gene expression studies where great differences were found between an *in vitro* (Arbillaga *et al.*, 2007a) and an *in vivo* analysis (Arbillaga *et al.*, 2008). Many of the pathways (mitochondrial electron transport chain, DNA damage response, MAPK signalling, Wnt signalling, RNA transcription, etc.) affected *in vitro*, were not affected *in vivo*, and the oxidative stress response that was mainly up-regulated *in vitro*, was down-regulated *in vivo*.

In conclusion, for the first time, different parameters related to oxidative stress have been measured following exactly the same methodological approach for both male and female F344 rats after 7 and 21 days OTA administration with doses known to produce tumours in 2-years bioassays. In accordance with the similar OTA levels and histopathological changes observed in the same animals (Pastor *et al.*, 2018) and with other oxidative-stress related parameters measured in both sexes (Hibi *et al.*, 2011), our results support that there are no differences between males and females in the oxidative stress response to OTA.

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Chapter 7

General overview

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1. Comet assay technical improvements

A wide variety of both endogenous and exogenous (*i.e.*, genotoxic) agents induce DNA damage in different ways, thereby providing the basis for mutations, which are strongly linked to cancer and other non-malignant diseases. As all mutagenic compounds are genotoxic, information on genotoxicity is of a key importance in the risk assessment process for different product classes to which humans may be exposed. In the food safety area, EFSA provided a Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011). In this document, an *in vitro* and *in vivo* step-wise approach was proposed for the generation and evaluation of data on genotoxic potential. In the suggested strategy, the test methods were referred (when available) by the corresponding harmonised guideline provided by the OECD. Although the OECD Guideline for the *in vivo* comet assay (OECD, 2016) was not developed at that time, the assay was considered for the *in vivo* follow-up of positive genotoxic findings *in vitro*.

The alkaline comet assay, first described as we use it nowadays in 1988 (Singh *et al.*, 1988), is a useful tool in several areas of research. Among other advantages, its relative simplicity and the possibility to apply it to any cell type (as long as a cell suspension can be obtained), together with a high sensitivity for detecting low levels of DNA damage, made it become so popular. However, beginnings were never easy. The high-variability due to protocol differences between laboratories made it necessary to identify and study those important factors affecting the results. Final agarose concentration in gels (Azqueta *et al.*, 2011; Ersson and Möller, 2011), duration of the alkaline unwinding treatment (Azqueta *et al.*, 2011; Ersson and Möller, 2011; Yendle *et al.*, 1997), electrophoresis conditions (Azqueta *et al.*, 2011; Ersson and Möller, 2011; Speit *et al.*, 1999; Vijayalaxmi *et al.*, 1992), enzyme-incubation time (Ersson and Möller, 2011), and DNA staining (Olive *et al.*, 1990) have been described as critical points influencing the outcome of the *in vitro* as well as the *in vivo* comet assays. On the other hand, although lysis conditions are also considered a critical variable (OECD, 2016), the influence of this parameter in the comet assay results has not been thoroughly studied. Several comet assay protocols recommended to lyse cells for at least 1 hour, being this time the most widely applied (Azqueta and Collins, 2013; Collins *et al.*, 2008; Tice *et al.*, 2000). However, 24-h and overnight lysis are also quite used (Azqueta and Collins, 2013).

With the purpose of assessing the influence of lysis conditions in the comet assay results, we evaluated (*in vitro*) the effect of modifying the time of lysis in untreated and MMS-, H_2O_2- or X-ray-treated cells on the alkaline comet assay results, as well as in untreated and Ro 19-8022

plus light-treated cells on the Fpg-modified comet assay results. In all the cases in which the standard comet assay was applied (*i.e.*, without enzymes), similar results were obtained either skipping the lysis step or after 1 h of lysis. In the case of the Fpg-modified comet assay, a 5-min lysis was necessary to allow Fpg to reach the nucleus. As previously reported in ɣ-irradiated lymphocytes, the lysis step could be even omitted when the standard comet assay was applied (Vivek Kumar *et al.*, 2009), as the alkaline treatment (a step forward in the comet assay protocol) is perfectly able to lyse the cells. However, except for X-ray-treated cells, our results showed an important increase in sensitivity for detecting DNA damage with longer times of lysis (*i.e.*, more than 1 h), without an increased DNA damage in untreated cells (Chapters 3 and 4). This observation might be due to the presence of some DNA lesions that could be spontaneously converted into AP-sites (*i.e.*, ALS) during the lysis period, and so detected as SBs with the alkaline comet assay (ALS are converted into breaks during the alkaline treatment).

In addition, we also checked the results obtained using two different lysis solutions; being one of them the most commonly used, and the other one prepared by adding N-Lauroylsarcosine sodium and DMSO (also very used). Similar results were obtained with both of them for every X-ray dose and time of lysis (Chapter 4).

Overall, the duration of the lysis step should be tightly controlled when performing both the *in vitro* and *in vivo* comet assays. A constant time of lysis should we used to reduce the inter-experimental and inter-laboratory variation, though using different times of lysis might be useful to increase the sensitivity and to ensure the detection of the DNA lesions induced by an unknown compound. Moreover, although more studies are needed to understand the underlying mechanism of the effect of the lysis time on the detection of different DNA lesions, varying the time of lysis could be used in the future to study the nature of induced DNA lesions.

Regarding the *in vivo* comet assay, an *In vivo* Mammalian Alkaline Comet Assay OECD Guideline (OECD, 2016) was finally achieved as a result of the first formal validation trial (Uno *et al.*, 2015a, 2015b). However, there are currently some specific limitations for this version of the assay; one of them has to do with the use of frozen tissues. It is often difficult from a logistical point of view to perform the *in vivo* comet assay in freshly-prepared tissues due to the high number of samples generated in a study. Thereby, freezing them for later analysis emerged as an alternative to solve this kind of difficulties when integrating the comet assay into repeated-dose toxicity studies (Recio *et al.*, 2012; Rothfuss *et al.*, 2011), or when combining it with the micronucleus assay (Recio *et al.*, 2010). Although the OECD guideline recognises that, in the literature, tissues or cell nuclei have been successfully frozen for later

comet assay analysis, it also requires the demonstration of the laboratory's proficiency in freezing methodologies (OECD, 2016). Moreover, currently there is no agreement on the best way to freeze and thaw tissues.

The comet assay has been applied to several frozen rodent tissue samples such as liver (*e.g.*, Folkmann *et al.*, 2007; Jackson *et al.*, 2013; Knudsen *et al.*, 2015; Løhr *et al.*, 2015; Risom *et al.*, 2007), kidney (*e.g.*, Knudsen *et al.*, 2015), lung (*e.g.*, Folkmann *et al.*, 2007; Jackson *et al.*, 2013; Knudsen *et al.*, 2015; Risom *et al.*, 2007), brain (*e.g.*, Forsberg *et al.*, 2015; Knudsen *et al.*, 2015) and spleen (*e.g.*, Knudsen *et al.*, 2015). Moreover, many of these studies performed the comet assay in combination with enzymes (*i.e.*, Fpg, Endo III or OGG1). Nevertheless, according to our knowledge, there is only one study demonstrating that similar results are obtained after performing the standard comet assay either in fresh or in frozen (liver and lung) tissues (Jackson *et al.*, 2013).

In the present work, different approaches (freezing/thawing combinations) were tested in liver tissues from untreated animals. Acceptable low % tail DNA values were obtained when small pieces inside a cryotube were flash frozen in liquid nitrogen, stored at -80°C and processed in a cold environment to prevent them to thaw until a cell suspension was obtained. Using the same approach, comparable results were obtained in fresh and 1-week or 1-month frozen liver, kidney and lung tissue samples from untreated or MMS-treated Wistar rats, with both the standard and the Fpg-modified comet assay (Chapter 5). Moreover, the group mean % tail DNA was lower than 6% for liver tissues of untreated animals in all cases (*i.e.*, fresh, 1-week and 1-month frozen tissues), as recommended by the OECD Guideline for liver tissues of vehicle-treated animals (it does not provide recommendation for other tissues) (OECD, 2016). The Fpg-modified comet assay is very used in genotoxicity testing, although it is not covered in the OECD Guideline, as necessary protocol modifications still need to be adequately characterised (OECD, 2016). Results showed that the thawing process seems to be crucial in preserving DNA integrity, and that up to 1-month-frozen liver, kidney and lung tissues can be used in genotoxicity testing. A stability study of frozen samples is currently ongoing at the Laboratory of Toxicology, and frozen samples from the same organs will be analysed after 3-, 6-months and 1-year storage at -80°C.

The inclusion of assay controls in each comet assay run, an approach to detect experiments with abnormal results due to technical issues, to assess inter-experimental variation and correct it, allowed to discard one of the experiments. These controls will be used to normalise the results after the completion of the stability study.

2. Application of the *in vivo* alkaline comet assay to the evaluation of the oxidative stress-response after OTA-treatment in F344 rats

The technical improvements for the *in vivo* comet assay enabled the application of the SOP to frozen kidney samples of a previous repeated-dose toxicity study of a naturally occurring food and feed contaminant (EFSA, 2006; WHO, 2008). OTA is a mycotoxin considered as one of the most powerful renal carcinogens in rodents and classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC) (IARC, 1993). Unfortunately, its mechanism of action is still unknown and several hypotheses have been postulated regarding this issue (WHO, 2008), being indirect DNA reactivity mediated by oxidative stress one of them. Moreover, large sex-differences have been observed in different carcinogenicity studies, being male rats more sensitive than females (Boorman *et al.*, 1992; Castegnaro *et al.*, 1998; NTP, 1989; Son *et al.*, 2003).

With this scenario, and using the samples obtained in a previous repeated-dose toxicity study with male and female F344 rats treated with 0 or 0.5 mg OTA/kg b.w. for 7 days, or with 0, 0.21 or 0.5 mg OTA/kg b.w. for 21 days, we applied both the standard alkaline and the Fpg-modified comet assay to frozen kidney samples of those animals to look for oxidative DNA damage. In addition, we also checked several oxidative-stress related parameters in kidney tissue of the same animals, mainly GST activity, tGSH and GSSG levels and SOD activity. No biologically-relevant differences due to OTA treatment or sex differences in the response to OTA treatment were found neither with the comet assay, nor with the different oxidative stress-related parameters.

3. Elaboration of Standard Operating Procedures (SOPs)

The Laboratory of Toxicology of The Department of Pharmacology and Toxicology of the University of Navarra is also part of the Drug Development Unit (DDUNAV) of the University. DDUNAV covers the different phases of drug development from preclinical to clinical phases (I, II, III and IV) for the purpose of offering the scientific knowledge of the University to companies, but in compliance with regulatory requirements. More specifically, the Laboratory of Toxicology offers technical development of new testing methods and diverse services related to preclinical toxicity testing, both *in vitro* and *in vivo*. The Unit offers it services to pharma, cosmetic, agro-food and emerging biotechnological companies, as toxicological studies can be carried out according to regulatory guidelines and Good Laboratory Practices (GLPs). In addition to regulatory compliant versions, versatile toxicity assays to be applied in basic research or at early stages of drug development are also offered.

The Laboratory of Toxicology is based in a center that has a Quality Assurance Unit and has a Certificate of Compliance with GLPs issued by the regional Government of Navarra in 1996, which was recently renovated (2016). Thereby, the Laboratory of Toxicology develops Standard Operating Procedures (SOPs) in order to apply them to studies under GLP conditions requested by external promoters.

In the present work, the knowledge derived from objective 1 (Chapters 3 and 4), regarding the time of lysis, was applied to the SOP for the *in vitro* alkaline comet assay. Therefore, a constant time of lysis within a set of experiments is recommended in that document. Furthermore, the SOP has been already approved by the Quality Assurance Unit, and thus the *in vitro* comet assay is currently offered as an external service for studies performed under GLPs.

On the other hand, the expertise gained in the application of the *in vivo* comet assay to both fresh and frozen tissue samples (Chapter 5) led to the elaboration of a SOP for the *in vivo* comet assay, which is currently being revised by the Quality Assurance Unit and will also be available soon.

Because of the public character of the present work, the aforementioned SOPs are not included due to confidentiality reasons.

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Chapter 8

Conclusions

Conclusions

1. With regard to **lysis conditions**:
   1.1. In the standard alkaline comet assay, the lysis step is not necessary to obtain the nucleoids since the alkaline treatment step is able not only to denature DNA but also to lyse the cells. In the case of the Fpg-modified comet assay, 5 min of lysis is enough to allow the Fpg to reach the nucleus.
   1.2. Increasing the time of lysis from 1 hour to 1 week highly increases the sensitivity of the assay depending on the DNA lesion detected.
   1.3. A constant time of lysis should be used in order to compare results from different experiments or laboratories.
   1.4. Adding N-Lauroylsarcosine sodium and DMSO to the commonly used lysis solution does not affect the standard comet assay outcome.
   1.5. Varying the time of lysis could be used in the future to study the nature of the DNA lesions induced; however, more studies are needed to understand the mechanism by which extra breaks are detected after applying long lysis periods.
2. With regard to tissue **freezing/thawing methods**:
   2.1. Acceptable low % tail DNA values, regarding both DNA SBs (plus ALS) and Fpg-sensitive sites, were obtained in frozen liver tissue from untreated animals when flash frozen as small pieces in liquid nitrogen, stored at -80°C and processed in a cold environment preventing the tissue samples to thaw until a cell suspension was obtained. This thawing process is of a key importance to avoid causing unintentional DNA damage.
   2.2. Frozen (for up to one month) liver, kidney and lung tissue samples, can be used in the *in vivo* comet assay (both with and without Fpg) for genotoxicity testing.
   2.3. The inclusion of assay controls in each comet assay run allows to detect technical problems and assess inter-experimental variability.
3. With regard to the **application** of the ***in vivo*** comet assay to kidney frozen samples from a previous **OTA repeated-dose study**:
   3.1. The new SOP developed for the comet assay was successfully applied, both with and without Fpg, to frozen kidney tissue samples obtained in a OTA repeated-dose toxicity study.
   3.2. No sex-differences, nor an increase in SBs or in Fpg-sensitive sites was found in kidney tissue samples of male and female OTA-treated F344 rats; this correlated to the

unaltered kidney oxidative-stress related parameters SOD and GST activities, and tGSH and GSSG levels, measured in the same samples.

4. With regard to the development of **SOPs** to be later applied in genotoxicity studies under **GLP conditions**:
   4.1. The use of a constant time of lysis within a set of comet assay experiments was included in the SOP for the *in vitro* comet assay.
   4.2. A SOP for the *in vivo* comet assay, both with and without Fpg, to be applied in fresh or frozen tissues, was developed.