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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. We have tested two different thawing procedures and compared the levels of DNA strand breaks (SBs) and Fpg-sensitive sites in fresh and frozen (for up to year) liver, kidney and lung tissue samples, from untreated and methyl methanosulfonate treated rats. Tissues were snap frozen, stored at −80 °C and processed in such a way that the tissue remained frozen until the cells were in suspension. Our results showed that comparable levels of DNA SBs were detected in fresh and frozen liver and lung samples stored at −80 °C for up to 1 year and 3 months, respectively. In kidney, similar levels of SBs were detected either in fresh or in frozen tissues stored for up to 1 year. However, more studies are needed to control the variability observed in the Fpg-sensitive site levels in this tissue at the different freezing periods.

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 and 2012 (Uno et al., 2015a, Uno et al., 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 Conference 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 (Brunborg et al., 2014; Pant et al., 2014). Moreover, it is advisable to integrate the *in vivo* comet assay into repeated-dose toxicity (RDT) studies (Rothfuss et al., 2010, 2011) or to perform it in combination with the micronucleus assay (Recio et al., 2010). Therefore, freezing of tissues for later analysis emerged as an option to overcome the logistic problems. Moreover, the use of freezing samples gives the opportunity to send the samples to a second laboratory to perform the comet assay analysis. 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). However, there is currently no agreement on the best way to freeze and thaw tissues (OECD, 2016).

The *in vivo* comet assay is also much used at the research level in academic institutions, where normally limited laboratory staff is available and therefore working with fresh samples is complicated. The possibility of working with frozen tissues is also a great advantage in this context.

Some researchers freeze tissue samples as a cell suspension using DMSO as a cryoprotectant (Hu et al., 2002; Recio et al., 2010, 2012; Pant et al., 2014; Kraynak et al., 2015). 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). The same group showed that liver, stomach and colon cells of untreated and ethyl methanosulfonate (EMS)-treated Sprague Dawley rats showed a significant increase in the % tail DNA in frozen samples stored for 1 or 8 weeks at −80 °C compared with fresh ones (Recio et al., 2012). Only the frozen stomach cells stored for 8

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weeks showed similar results as the fresh ones. However, the background levels of damage in fresh and frozen liver and stomach samples were within the acceptance range established by JaCVAM (i.e., 1–8% for liver and 1–20% for stomach) (Uno et al., 2015a and Uno et al., 2015b) and a dose response was observed in all cases. 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. In the case of the male frozen liver, the obtained levels were outside their fresh liver historical control ranges and above the recommended optimal range. However, freezing female liver, and male or female stomach cell suspensions had no effect on the background % tail DNA (Pant et al., 2014). Cell suspensions were flash frozen using 10% DMSO in these three studies, but the thawing process was done in a different way. Recio et al. (2010) stated that samples were thawed at room temperature and left to allow the large pieces to settle before taking the supernatant for the comet analysis. In their more recent study samples were partially thawed at room temperature but then placed on ice for few seconds to allow large pieces to settle (Recio et al., 2012). In the case of the study of Pant et al. it is not clear how they perform the thawing of the samples (Pant et al., 2014).

Rapid thawing (in a 37 °C water bath) and quick processing of frozen liver cell suspensions of untreated, 2-acetylaminofluorene (AAF)- or cisplatin (CPN)-treated Sprague-Dawley rats, gave comparable results to those obtained with fresh preparations (Kraynak et al., 2015). However, levels of DNA SBs (and ALS) induced/detected in treated rats were very low in all cases (i.e. < 3.5% DNA in tail aprox.). On the other hand, fresh and frozen liver from the AAF-treated cells were obtained in different studies. In these studies, results obtained in fresh and frozen liver samples from animals administered with EMS (used as positive control) showed similar and a bit higher % DNA in tail (i.e., 14.9 ± 1.0% in fresh samples and 15.4 ± 1.7% in frozen ones).

Hu et al. (2002) showed no significant differences in DNA SB levels measured as tail moment in fresh and frozen liver and kidney tissues of untreated and ferric nitriloacetate (Fe/NTA)-treated Sprague-Dawley rats. In this study, samples were thoroughly minced, frozen at −85 °C in PBS and 10% DMSO, and stored for 72 h. On the day of the analysis, tissues were thawed in a bath at 37 °C (for 2–3 min), the cryprotectant solution was removed, and the tissues were digested with collagenase for 10 min at 37 °C.

Freezing of tissues as cell suspensions may be a good option, or even the only one depending on the tissue (*e.g*, in the case of the glandular stomach or colon tissues, scraped epithelial cells are used for the comet assay analysis). However, this approach might not be optimal in order to overcome the logistical problems due to the handling of a huge number of samples, as it already involves many time-consuming steps.

In the literature, the comet assay has been applied to several frozen rodent tissue samples such as liver (*e.g.*, Risom et al., 2007; Folkmann et al., 2007; Jackson et al., 2013; Knudsen et al., 2015; Løhr et al., 2015), kidney (*e.g.*, Knudsen et al., 2015) lung (*e.g.*, Risom et al., 2007, Folkmann et al., 2007; Jackson et al., 2013; Knudsen et al., 2015), brain (*e.g.*, Forsberg et al., 2015; Knudsen et al., 2015) and spleen (*e.g.*, Knudsen et al., 2015). Moreover, many of these studies involved the comet assay in combination with enzymes (Formamidopyrimidine-DNA glycosylase, Fpg; endonuclease III, Endo III; or 8-oxo-Gua DNA glycosylase, OGG1). It is worth mentioning that the enzyme-modified comet assay is not included in the OECD guideline 489 (OECD, 2016). Small tissue samples are most commonly snap frozen in liquid nitrogen and stored at −80 °C until further processing (Risom et al., 2007; Folkmann et al., 2007; Jackson et al., 2013; Knudsen et al., 2015; Løhr et al., 2015; Forsberg et al., 2015).

According to our knowledge, Jackson and colleagues have been the only group that has thoroughly described both the freezing and thawing process (i.e. 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. Results showed that snap freezing in liquid nitrogen of small pieces (*i.e.*, 3 × 3 × 3 mm) of tissue (previously placed in cryotubes), in combination with the disaggregation 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 sample to thaw at room temperature yielded very high levels of SBs. Moreover, this is the only work in which the comparison between fresh and immediately frozen tissues has been done; 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.

In this work, we have tested the suitability of two different thawing methods, applied to flash frozen liver tissues from untreated rats, to carry out both the standard and the Fpg-modified comet assay. In addition, a stability study of frozen tissues has been carried out by comparing the levels of both DNA SBs (plus alkali labile sites, ALS) and Fpg-sensitive sites (*i.e.*, altered purines) in fresh and frozen liver, kidney and lung tissues, from control and MMS-treated rats, stored for different times (up to 1 year) in −80 °C. Two different doses of MMS were used in order to produced detectable DNA SBs and Fpg-sensitive sites.

2. Materials and methods

2.1. Chemicals and reagents

Low melting point agarose, standard agarose, Triton X-100, Tris base, HEPES, Na_2EDTA, Bovine Serum Albumin (BSA), MMS and 4',6-diamidino-2-phenylindole (DAPI) 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). All cell culture reagents were purchased from Gibco.

2.2. Animals

For testing the two different thawing methods on flash frozen liver tissues, untreated animals 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 3Rs (Reduce, Refine and Replace) strategy.

For the stability study, 15 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.

One group of animals (n = 5) was orally administered with a single dose of 5 mg/kg b.w. MMS to induce lesions that can be detected with the Fpg-modified comet assay (oxidized and alkylated lesions) without inducing DNA SBs; another group (n = 5) was orally administered with a single dose of 200 mg/kg b.w. MMS to induce DNA SBs, and a third group (n = 5) was not treated (negative control group).

For the administration regimen, one animal of each group was orally administered with 5 or 200 mg/kg b.w. MMS, or nothing on five consecutive days. 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 obtention and cryopreservation

The procedure followed to obtain the samples was the following. Organs were extracted at necropsy, washed in Merchant's medium, transferred to a Petri dish on ice and cut to obtain 3 × 3 × 3 mm (aprox.) liver and lung pieces or a piece of similar volume of kidney (2 × 3 × 5 mm, aprox.). Kidney was cut in pieces containing medulla and cortex in each sample. Immediately afterwards one sample was prepared to analyse fresh and several other samples were transferred into cryotubes, flash frozen in liquid nitrogen and stored at −80 °C.

2.4. Sample processing: testing thawing procedures

In order to test the best thawing procedure liver pieces from untreated rats from previous toxicity studies were processed. They were thawed for analysis using 2 procedures:

1.- A sample was immediately put on ice, washed in a beaker with ice-cold 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 Na2EDTA, pH 7.4), transferred to another beaker with ice-cold Merchant's medium, cut into pieces with scissors, homogenised using a cylindrical metal sieve (tissue mincing device, NorGenoTech) by moving a plastic plunger (from 1 mL syringe) up and down several times, filtered through a 100 μm nylon filter, centrifuged for 5 min at 214 × *g* and 4 °C and cell pellet suspended in Merchant's medium (centrifugation and suspension was carried out twice), before starting the comet assay.

2.-Following the procedure described by Jackson et al. (2013) to thaw samples, a cryotube containing the sample was placed on dry ice and a drop of Merchant's medium was placed, using a Pasteur pipet, on top of the tissue to create a protective ice cap. The deep-frozen tissue was then transferred, using dry ice-cold tweezers, into the cylindrical metal sieve (tissue mincing device, NorGenoTech). The sieve was previously immersed in a plastic tube containing 3 mL Merchant's medium on ice. The tissue was then homogenised by moving a plastic plunger (from a 1 mL syringe) up and down several times, forcing it to pass through the sieve. The homogenised samples were collected in the 3 mL Merchant's medium. It is important to ensure that the plastic tube containing the Merchant's medium, in which the sample is collected, is kept on ice all the time.

The critical difference between the two methods is that in method 1, the sample thaws before homogenising, while in method 2 the sample is still frozen when starting the homogenisation.

The standard and the Fpg-modified comet assay were applied in these tissues.

2.5. Stability study

Fresh samples were processed as follows: a piece of tissue was transferred to another Petri dish on ice and thoroughly dissected using a scalpel. The minced tissue was then transferred to a tube containing 2 mL of Merchant's medium on ice, using a Pasteur pipet, and further disaggregated by making it go through the pipet several times (up and down). In this way a cells suspension was obtained and maintained on ice until a straightforward comet assay analysis.

All frozen samples were processed after 1 week, 1, 3 and 6 months, and 1 year, following the procedure number 2 described in the previous section (2.4. Sample processing: testing thawing procedures) before the comet assay analysis.

2.6. Assay controls for the comet assay

TK-6 cells were used to produce positive and negative assay controls for the standard and the Fpg-modified comet assay.

TK-6 cells, derived from a human lymphoblastoid cell line, were obtained from the American Type Culture Collection (ATCC) and grown in RPMI medium with with 2 mM L-glutamine supplemented with 10% heat-inactivated foetal calf serum, 0.2 mg/ml sodium pyruvate, 100U/ml penicillin and 0.1 mg/ml streptomycin (all from Gibco). Cells were maintained as a suspension culture at 37 °C in a humidified atmosphere with 5% CO_2. Cell were used after 1 week and no more than 2 months in culture.

Positive assay controls were produced by treating TK-6 cells with a) 300 μM MMS for 3 h to induce DNA SBs (positive control for the standard comet assay) or b) 50 μM MMS for 3 h to induce Fpg-sensitive sites (positive control for the Fpg-modified comet assay). Untreated cells were used as negative assay controls.

These assay controls were prepared one week in advance of each time point of tissue analysis (1, 3 and 6 months, and 1 year), except for the analysis of fresh and 1-week frozen samples, in which case exactly the same controls that had been obtained the week before obtaining the samples, were used. Cells were treated, washed by centrifugation and frozen in aliquots. Inclusion of these controls allows discarding experiments in case of abnormalities in the results due to technical problems.

2.7. Comet assay

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 and rats treated with 5 or 200 mg/kg b.w. of MMS). In addition, the Fpg-modified comet assay 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.

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 × 20 mm coverslips. Gels were set on a metal plate on ice for 3 min and the coverslips were removed. In cases where the standard and the Fpg-modified comet assay were applied, three slides were prepared and further processed per condition: 'lysis', 'buffer F' and 'Fpg'. In the case of the standard comet assay only the 'lysis' slide was prepared and processed.

Overnight lysis at 4 °C was performed by immersion of the slides 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 (buffer F) (40 mM HEPES, 0.1 M KCl, 0.5 mM, Na_2EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0) at 4 °C. Afterwards, gels were incubated with buffer F or Fpg by adding a drop of 45 μL. Each drop was covered with a 22 × 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 washing 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 ALS are measured in the 'Lysis' slide, while net Fpg-sensitive sites were calculated by subtracting the median value of the 'Buffer F' slide from that obtained in the 'Fpg' slide.

Positive and negative assay controls were included in each electrophoresis run to assess the correct performance of the assay and the inter-assay reproducibility. Moreover, a sample of each organ from untreated and MMS-treated animals was included in each experiment.

Table 1
Mean and SD of the levels of DNA SBs and Fpg-sensitive sites (% tail DNA) obtained in the assay controls with TK-6 cells included in all the experiments performed with fresh and frozen samples at the different times points.

			Frozen samples				
		0 (Fresh)	1 week	1 month	3 months	6 months	1 year
Number of experiments		6	6	5 (#)	4 (##)	6	6
Untreated	SBs + ALS	1.7 (2.4)	1.5 (0.9)	0.6 (0.7)	0.2 (0.1)	0.6 (0.9)	0.2 (0.4)
	Net Fpg-sensitive sites	0.3 (0.5)	2.2 (1.5)	1.33 (1.3)	1.8 (0.4)	0.7 (0.8)	0.5 (0.9)
50 μM MMS	SBs + ALS	6.1 (2.3)	6.3 (2.2)	7.5 (2.7)	5.5 (1.6)	4.1 (1.7)	2.1 (1.5)
	Net Fpg-sensitive sites	67.7 (8.6)	65.9 (8.7)	53.5 (11.3)	– (###)	72.4 (11.1)	45.8 (13.9)
300 μM MMS	SBs + ALS	42.2 (7.1)	47.2 (10.0)	56.5 (8.4)	50.9 (6.6)	47.2 (5.5)	43.9 (3.0)

(#) Very high values were obtained in 1 of the experiments (out of 6) so the whole experiment was discarded.
(##) Very high values were obtained in 2 of the experiments (out of 6) so the whole experiments were discarded.
(###) all experiments performed with the Fpg were discarded due to problems with the buffer F giving high values of % DNA in tail in some cases.

Six experiments were performed at each time point to analyse all the samples.

2.8. Statistics

Mean and SD were calculated per tissue and time point (i.e., fresh and frozen tissues for the different periods). Non-parametric Kruskal-Wallis was used to compare the levels of DNA SBs + ALS and Fpg-sensitive sites of the different organs at the different time points of storage (including fresh samples). Whenever statistical significance was observed, the Dunn's multiple comparison test was applied to compare results obtained at the different time points of storage with the values of the fresh tissues. Kruskal-Wallis and the Dunn's multiple comparison tests were performed using Graph Pad.

The non-parametric Mann-Kendall test was applied to detect a monotonic trend using Excel.

3. Results

Highly damaged comets, not even possible to be accurately measured (i.e., more than 90% DNA in tail), were obtained after applying the standard comet assay in frozen liver tissues from untreated animals when using the thawing procedure number 1 (2.4. Sample processing: testing thawing procedures). Due to this, the Fpg-comet assay could not be applied. However, following the thawing procedure number 2 (2.4. Sample processing: testing thawing procedures), and after analysing 6 samples, the level of SBs + ALS was 4.3 ± 3.6% DNA in tail. The level of net Fpg-sensitive sites, obtained after analysing 2 samples, was 10.4 ± 2.6%. Therefore, procedure number 2 was selected for further analysis.

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, 1, 3 and 6 months, and 1 year, following the procedure described in the material and methods section. Assay controls were included in each of the experiments. As explained before, they were prepared, and frozen, 1 week in advance of the correspondent time point of analysis. Same assay controls were used in the experiments in which fresh and 1 week frozen tissues were analyzed. Obtained results in assay controls are presented in Table 1.

Assay controls from 3 experiments in which some 1-month-frozen samples (1 experiment) and 3-month-frozen samples (2 experiments) were analysed, showed a high deviation from the expected % DNA in tail. These experiments were discarded due to obvious technical problems. Moreover, the Fpg-modified comet assay could not be applied in 3-months-frozen tissues from animals treated with 5 mg MMS/kg b.w.

Results obtained using the standard and the Fpg-modified alkaline comet assay in fresh and frozen liver, kidney and lung samples of untreated animals are shown in Fig. 1. As can be observed, low levels of SBs + ALS and Fpg-sensitive sites were obtained in all tissues and at all the different conditions tested (i.e., fresh and frozen tissues for the different periods). When looking at the statistical analysis, significant differences were observed between the levels of SBs + ALS of fresh (3.4 ± 1.4%) and 3-months- (0.8 ± 0.5%) and 1-year- (1.0 ± 1.2%) frozen liver samples; and also between fresh (5.6 ± 1.7%) and 1-year- (1.3 ± 1.5%) frozen kidney samples. The level of Fpg-sensitive sites in liver was similar at the different concentrations tested. However, in kidney, although significant differences were observed between the different conditions tested, when applying the Kruskal-Wallis test, no

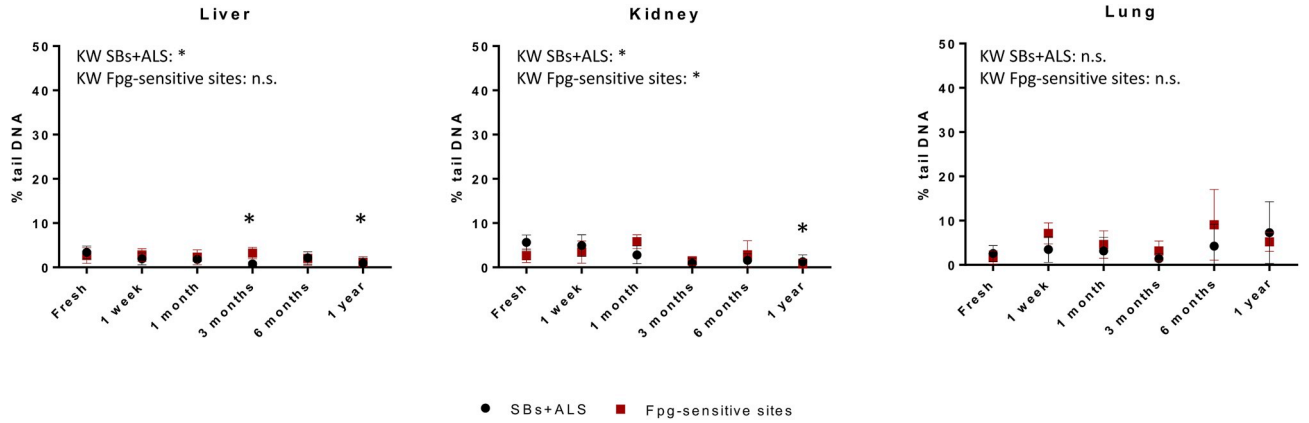

Fig. 1. Mean and SD of the levels of DNA SBs and Fpg-sensitive sites (% tail DNA) found in fresh, 1 week-, 1 month-, 3 months-, 6 months- and 1 year-frozen (at −80 °C) liver, kidney and lung tissue samples from untreated animals. Results of the Kruskal-Wallis (KW) test are shown on the left corner of each panel. *: level of SBs + ALS significant vs fresh samples (Dunn's test, p-value ≤ 0.05).

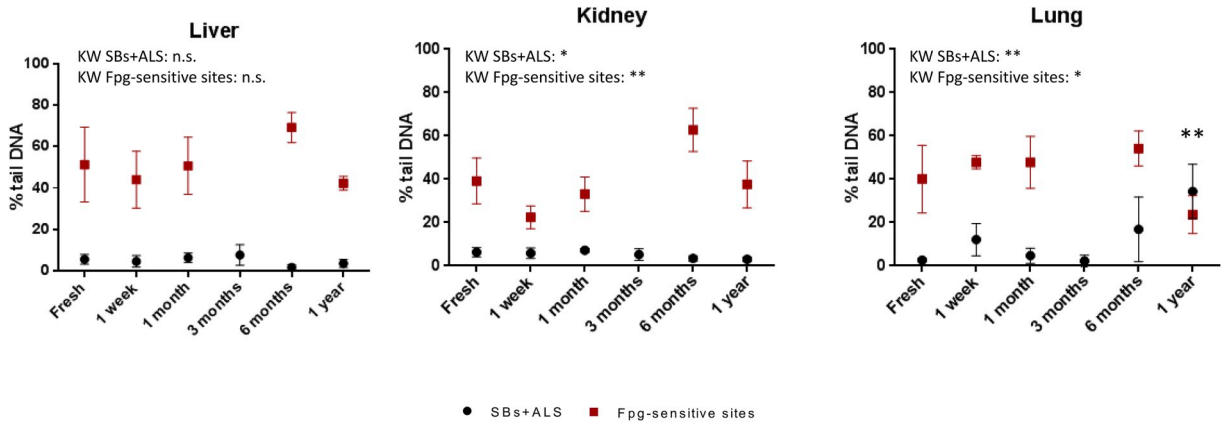

Fig. 2. Mean and SD of the levels of DNA SBs and Fpg-sensitive sites (% tail DNA) found in fresh, 1 week-, 1 month-, 3 months-, 6 months- and 1 year-frozen (at −80 °C) liver, kidney and lung tissue samples from rats treated with 5 mg MMS/kg b.w. Results of the Kruskal-Wallis (KW) test are shown on the left corner of each panel. **: level of SBs + ALS very significant vs fresh samples (Dunn's test, p-value ≤ 0.01).

significant differences were found between fresh and frozen samples. In the case of the lung tissues, no statistically-significant differences were detected, but a slight increase and a high inter-sample variability in the level of SBs + ALS was observed in 6-months- and 1-year-frozen tissues (i.e. fresh tissue: 2.6 ± 1.8%, 6-month-frozen tissue: 4.2 ± 4.9%, 1-year-frozen tissue 7.3 ± 7.0%). A slight increase and high variability of the net Fpg-sensitive sites were only observed in 6-months-frozen tissues. In any case, all the observed values can be considered within the normal range for control values.

The levels of DNA SBs + ALS and Fpg-sensitive sites of liver, kidney and lung tissue samples from animals treated with 5 mg MMS/kg b.w. are shown in Fig. 2. The levels of SBs + ALS observed were low in liver and kidney tissues in all conditions tested; no differences were observed in liver samples while statistically-significant differences appeared in kidney when applying the Kruskal-Wallis test. Moreover, no differences were found between fresh and frozen kidney samples. In the lung tissues, a very significant increase was observerd in 1-year-frozen samples (34.4 ± 12.5%) compared to fresh samples (2.6 ± 0.8%). A slight increase (not statistically significant) was observed at 6 months (16.9 ± 14.9%). As can be observed a high inter-sample variability was observed at these two timepoints.

The levels of net Fpg-sensitive sites obtained in fresh samples were similar in the 3 tissues analysed (Fig. 2). Non-significant differences were observed in the liver tissues at the different conditions tested (Fig. 2). In kidney, the Kruskal-Wallis test showed significant variation among the different conditions, but a direct comparison of fresh with each frozen sample by Dunn's test showed no significant differences. Moreover, very similar results were observed in fresh (39.2 ± 10.6%) and 1 year-frozen samples (37.6 ± 10.8%).

In the lung tissues, significant differences were observed between the different conditions tested when applying the Kruskal-Wallis test. The decrease seen at 1 year was not significant by the Dunn's test.

In the animals treated with 200 mg MMS/kg b.w., no differences were observed in the level of DNA SBs + ALS in liver, kidney and lung tissues, at the different conditions tested (i.e., fresh and frozen samples during different periods) (Fig. 3). An increase was observed in 1 year-frozen lung tissues compared with fresh ones, although not statistically significant.

A monotonic trend, evaluated by the Mann-Kendall test, was not observed in any of the tissues and at any of the concentrations tested.

4. Discussion

In 2016, the OECD published the current version of the guideline to perform the comet assay in several animal tissues (OECD, 2016). This guideline is focused on the use of fresh tissues though it contemplates the possibility to work with frozen nuclei or tissues. In this case, the laboratory should first demonstrate competency since nowadays there is no agreement about how to freeze and thaw frozen nuclei or tissues.

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 not maintained fresh (Knudsen et al., 2005; Guerard et al., 2014), 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 a constant and acceptable length of time for the preparation of the

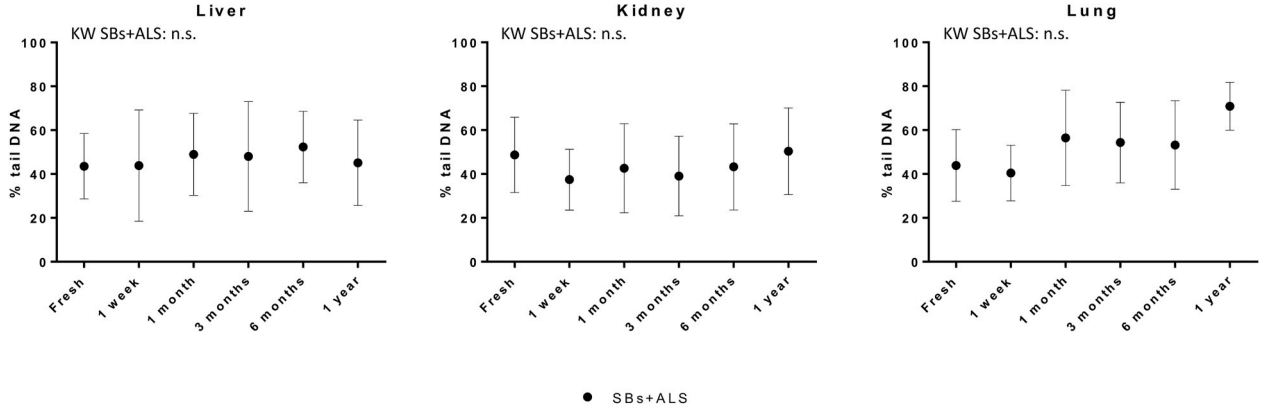

Fig. 3. Mean and SD of the levels of DNA SBs (% tail DNA) found in fresh, 1 week-, 1 month-, 3 months-, 6 months- and 1 year-frozen (at −80 °C) liver, kidney and lung tissue samples from rats treated with 200 mg MMS/kg b.w. Results of the Kruskal-Wallis (KW) test are shown on the left corner of each panel.

specimens, thereby helping to reduce variation due to the processing of numerous samples.

As was mentioned in the introduction, the standard and the Fpg-modified alkaline comet assay have been applied to several frozen rodent tissue samples and different approaches have been used to freeze/thaw the samples. However, only Jackson et al. thoroughly described a protocol to freeze and thaw the samples to perform the alkaline comet assay in lung and liver (Jackson et al., 2013). In this work we have used the same protocol to perform the standard and the Fpg-modified alkaline comet assay in liver, kidney and lung, and we have studied the stability of the frozen samples for up to 1 year at −80 °C.

To set up the freezing/thawing procedure we performed the standard alkaline comet assay in liver samples, from untreated animals, thawed by using two different approaches, the one described in Jacskon et al. being the successful one (Jacksons et al., 2013). This approach avoids the tissue thawing until a cell suspension is obtained in a cold environment on the day of comet analysis. After analysing 6 samples the level of SBs + ALS was 4.3 ± 3.6% DNA in tail, meeting the criteria of the *In Vivo* Mammalian Alkaline Comet Assay OECD Guideline in which it is stated that the level of SBs + ALS in liver from untreated animals should not exceed 6% DNA in tail (OECD, 2016). The Fpg-modified comet assay was also successfully applied in frozen samples in which thawing was avoided until getting the cell suspension; the level of net Fpg-sensitive sites obtained after analysing 2 samples was 10.4 ± 2.6%, an acceptable level for tissues from an untreated animal.

Although data are not shown, we also tested the pulverization (by giving a single sharp impact with a hammer after placing the tissue in a dry ice-chilled metal pulverizer, avoiding any grinding process) of liver samples from untreated animals This procedure also worked perfectly though a lot of care must be taken in maintaining the pulveriser dry and dry ice-cooled, cleaning it very well between samples and avoiding its moistening. Analysing different frozen samples at one run can be complicated.

The other thawing approach was to thaw the tissues completely, though maintaining them ice-cold, and processing them as with fresh samples. In this case, high levels of SBs + ALS were observed (more than 90% DNA in tail). Jackson et al. also showed that the thawing of the tissues gave very high levels of SBs (Jacksons et al., 2013). They showed that freezing big pieces of tissue and, afterwards, cutting or crushing them before being processed significantly increased DNA SBs. Moreover, 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.

The successful protocol of thawing was applied to frozen liver, kidney and lung samples from untreated rats or rats administered with 5 (to induce Fpg-sensitive sites) or 200 (to induce SBs) mg MMS/kg b.w. by oral gavage. Samples were analysed after 1 week, 1, 3 and 6 months, and 1 year, of being snap frozen in liquid nitrogen and stored at −80 °C. The standard and the Fpg-modified alkaline comet assay were applied to tissues from untreated and 5 mg MMS/kg b.w. treated animals, while only the standard version was applied to tissues from 200 mg MMS/kg b.w. treated animals. Although both versions of the assay have been applied to frozen tissues from different organs in several occasions, comparison between fresh and frozen tissues is scarce. According to our knowledge, only Jackson et al. compared the levels of SBs in fresh and frozen liver and lung tissue from untreated and MMS-treated mice (Jackson et al., 2013).

According to our results, frozen liver samples for up to 1 year, from untreated rats or rats administered with the different doses of MMS, gave comparable levels of SBs + ALS and Fpg-sensitive sites to those in fresh samples. Some statistical differences appeared in the levels of SBs + ALS in samples from untreated animals; however, the biological meaning of these differences is debatable since the levels of SBs + ALS were very low in all the cases (< 3.5% tail DNA). In this regard, all fresh and frozen samples meet the criteria of the OECD about the acceptance level of SBs + ALS in liver from untreated animals, which should not exceed 6% DNA in tail (OECD, 2016). Jackson et al. compared the level of SBs + ALS of fresh and frozen liver tissues from untreated and MMS-treated mice (25, 75 and 112.5 mg MMS/kg b.w.) (Jackson et al., 2013). They also found that SBs + ALS in fresh and frozen liver tissues from untreated and MMS-treated mice were similar but, unfortunately, the time frozen samples were kept at −80 °C was not specified. According to our result, the level of SBs + ALS and Fpg-sensitive sites can be measured in frozen liver samples stored for 1 year at −80 °C.

In the case of the kidney, comparable levels of SBs + ALS and Fpg-sensitive sites were obtained in fresh and frozen samples for up to 1 year in untreated animals. As mentioned for the liver tissues, some statistical differences observed in the level of the SBs and Fpg-sensitive sites may not be biologically relevant due to the low level of these DNA lesions (< 5% tail DNA for both SBs and Fpg-sensitive sites). The same situation occurs with the level of SBs detected in kidney from animals treated with 5 mg MMS/Kg b.w. However, the levels of Fpg-sensitive sites in these tissues vary at the different time points though they are not statistically different from the fresh tissues. From a technical point of view, it is difficult to explain this high variability among the different time points only in the kidney, as all three organs were analysed together. However, it should be taken into account that kidney is a heterogeneous organ, with many cell types. Thus, it could be hypothesized that small variations in the experiment might affect much more the kidney than other organs. However, we consider that analysing the robustness and therefore the capacity of the method to remain unaltered under small but deliberate variations (for instance, ambient temperature, freezing/thawing times, time of samples on the bench) during tissue sample and cell suspension preparation might shed some light on the interpretation of these results.

Lung samples from untreated animals, stored at −80 °C for up to 1 year, show similar levels of SBs + ALS and Fpg-sensitive sites as fresh samples, though a slight increase (not statistically significant) of SBs + ALS was observed in 6-months- and 1 year-frozen samples. This increase was more pronounced in tissues from animals treated with 5 mg MMS/kg b.w., and was very significant in the case of the 1-year-frozen sample compared to fresh samples. Moreover, the increase was accompanied by a non-statistically significant decrease, compared with the fresh samples, in the level of Fpg-sensitive sites, probably due to the conversion of the Fpg-detected lesions into breaks. In the case of levels of SBs + ALS observed in animals treated with treated with 200 mg MMS/kg b.w., there were not significant differences between the fresh and the frozen samples but an increase (not statistically significant) was observed in 1-year-frozen samples. These results indicated that DNA from lung tissue might suffer some degradation during the long storage as frozen sample; it would be then advisable to analyse the frozen lung tissues, to detect both SBs + ALS and Fpg-sensitive sites, within 3 months. Jackson et al. also found a slight but significant increase in frozen lung tissues from untreated mice when compared with the fresh ones (i.e., from 2.9 to 7.1% DNA in tail) (Jackson et al., 2013). However, the time of the storage of the frozen samples was not specified in the paper.

It should be noted that in the present study only 100 comets were counted per slide; while the OECD recommends counting at least 150 comets per tissue and animal (OECD, 2016). This would probably have helped to reduce the observed variability, especially in levels of Fpg-sensitive sites of kidney.

Assay controls, consisting of in untreated and MMS-treated TK-6 cells (50 μM MMS was used to induce Fpg-sensitive sites, while 300 μM MMS was used to induce SBs + ALS), were included in each experiment to assess the technical variability of the assay (inter-experimental variation). These assay controls were included in order to detect technical problems and actually, they allowed us to discard 3 experiments in which the levels of DNA lesions in the assay controls were unexpected (accompanied with unexpected results in the tissues).

Assay controls presenting a modest variation, can also be used for the normalization of the data and therefore to remove, at least partially, the technical inter-experimental variation (Collins et al., 2014). To do so, the same batch of assay controls should be prepared, frozen and included in each experiment. In our case, and since we have not tested the long term stability of the frozen assay controls, they were prepared one week in advance of each time point. Due to this, normalization of the data could not be performed.

The enzyme-modified comet assay is not covered in the OECD guideline 489 (OECD, 2016). Though it mentions the possibility of using the comet assay to measure oxidized bases (this is done by the combination of the comet assay with lesion specific enzymes), it also states that the necessary protocol modifications still need to be adequately characterised (OECD, 2016). The Fpg-modified comet assay has been shown to increase the sensitivity of the *in vitro* comet assay (Azqueta et al., 2013) and it also could be a good tool to increase the sensitivity of the *in vivo* comet assay. As is shown in this work, the inclusion of the Fpg allows us to detect DNA lesions at an MMS dose (i.e. 5 mg MMS/kg b.w.) that does not induce SBs + ALS (Fig. 2). However, the protocol should be further analysed, at least for some tissues. The alkaline comet assay in combination with Fpg detects not only oxidized purines but also some alkylated lesions, but it is worth mentioning that other enzymes can be used to detect different DNA lesions (Azqueta and Collins, 2013).

In 2011, a working group convened at the 5th IWGT (International Workshop on Genotoxicity Testing) to discuss the improvement of the *in vivo* genotoxicity testing (by the combination of genotoxicity assessment in standard toxicity tests), concluded that freezing samples for the comet assay analysis could not be recommended for routine used (Rothfuss et al., 2011). However, they pointed out that frozen samples can be used if the laboratory demonstrates that reproducible results with frozen and fresh tissues are obtained. They showed results obtained using fresh and frozen liver samples from animals treated with clofibrate or different concentrations of 1,2-dibromomethane, acrylamide or gemifloxacin. Results are shown as a fold increase of % tail intensity and authors claimed a decrease in the comet assay response when frozen tissues are used. It is not clear enough how they calculated the fold increase but we guess that it was done by comparing the results in samples from treated animals to the ones in samples from untreated animals. If this is the case, the impact of the basal damage (i.e. from untreated animals) when calculating a fold increase is tremendous. As an example, a basal damage of 1% or 2% DNA in tail, both acceptable and similar values, will give very different fold-change increase even when a similar dose response is obtained when testing a chemical. Moreover it is very difficult by using the fold-change to know the basal damage in the untreated samples.

A few years later, Speit et al. published a report about the critical points of the *in vivo* comet assay in regulatory genotoxicity testing (Speit et al., 2015). The expert working group met in the 6th IWGT. They affirmed that more data/experience are needed for the use of frozen tissues. Moreover, they pointed out the need for a generally accepted protocol for the acceptability of the results obtained using frozen tissues. Our study contributes to gaining experience and knowledge about the use of frozen tissues for comet assay analysis and may contribute to create a generally accepted protocol.

As 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 tissue DNA integrity. Therefore, 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. Frozen liver samples, stored for up to 1 year at −80 °C, can be used to assess the level of both SBs + ALS and the Fpg-sensitive sites, in samples from untreated and treated animals. Similarly, frozen kidney samples can be used to measure SBs + ALS, though more studies are needed to control the variability observed in the Fpg-sensitive sites. In the case of frozen lung tissues, they can be safely processed, to measure both SBs + ALS and Fpg-sensitive sites, after storing them for up to 3 months.

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Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

All the authors (Azqueta Amaya, Enciso Jose Manuel, Pastor Laura, López de Cerain Adela, Vettorazzi Ariane) have nothing to disclose.

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