Collection and storage of human white blood cells for analysis of DNA damage and repair activity using the comet assay in molecular epidemiology studies

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Abstract

DNA damage and repair activity are often assessed in blood samples from humans in different types of molecular epidemiology studies. However, it is not always feasible to analyse the samples on the day of collection without any type of storage. For instance, certain studies use repeated sampling of cells from the same subject or samples from different subjects collected at different time-points, and it is desirable to analyse all these samples in the same comet assay experiment. In addition, flawless comet assay analyses on frozen samples opens up for the possibility of using this technique on biobank material. In this article we discuss the use of cryopreserved peripheral blood mononuclear cells (PBMCs), buffy coat (BC) and whole blood (WB) for analysis of DNA damage and repair using the comet assay. The published literature and the authors' experiences indicate that various types of blood samples can be cryopreserved with only minor effect on the basal level of DNA damage. There is evidence to suggest that WB and PBMCs can be cryopreserved for several years without much effect on the level of DNA damage. However, care should be taken when cryopreserving WB and BCs. It is possible to use either fresh or frozen samples of blood cells, but results from fresh and frozen cells should not be used in the same dataset. The article outlines detailed protocols for the cryopreservation of PBMCs, BCs and WB samples.

Introduction

Analysis of DNA damage, DNA repair and sensitivity to genotoxic exposure can be carried out on fresh or frozen samples of cells [1;2]. In most laboratories, processing of fresh samples is the method of choice because possibly deleterious effects of the freezing and thawing processes are thus avoided. In fact, detailed comet assay protocols on fresh cells and tissues, with numerous technical notes and recommendations, have been published over the years, but they do not describe the best practice for cryopreservation and thawing of samples (summarized by Azqueta et al. [3]). It is not always possible to analyse all samples in the same comet assay experiment and sometimes it is wiser to cryopreserve samples for later analysis. One example is field studies where specimens are collected outside the laboratory and immediate processing is not feasible. In this case, researchers have to consider the pros and cons of the impact of storage at ambient temperature and cryopreservation [4]. Many comet assay researchers use equipment that has medium or low throughput (typically not more than 20 samples per experiment), which puts a limit to the number of samples that can be analysed per experiment. Thus, it may be necessary to use cryopreserved samples as internal references to control for inter-assay variation. However, there appears to have been a tendency not to include assay controls in biomonitoring studies [5;6], which may be due to uncertainty about the stability of DNA damage levels in cryopreserved samples. Another area is biobank material, collected in large cohorts, which may be useful for comet assay research if it can be confirmed that storage in the freezer for a long period of time and subsequent thawing does not affect the DNA damage level or repair activity of the sample. **Table 1** lists advantages and limitations of cryopreservation of samples for the comet assay.

Comet assay researchers have experimented with cryopreservation of samples for at least three decades, building mainly on the knowledge from cryopreservation techniques used for storage of cell cultures. Nevertheless, there is an important difference between the aim of

cryopreserving samples for later cell culture and comet assay analysis. For the latter, it is important to minimise DNA breakage, but the cells do not need to be viable. In contrast, cryopreservation for later culture of cell lines aims at increasing the viability of the thawed cells. This procedure can afford the luxury of having a proportion of the cells that are too damaged to establish a cell culture. However, this is not possible for samples that are cryopreserved for the comet assay because all cells are analysed and damaged cells will have high background level of DNA migration. The survival rate of thawed cells depends on the cell type, but many cell biologists would consider a survival rate of 90% to be a success. Dead or dying cells typically give rise to comets with long tails and it will have a strong impact on the mean level of DNA migration if only 10% of the cells were damaged. For instance, a sample with 5% tail DNA before cryopreservation will have mean level of 12% tail DNA after thawing if 10% of the cells are highly damaged [(90 cells × 5% tail DNA + 10 cells × 75% tail DNA)/100 cells = 12% tail DNA; assuming that highly damaged cells have 75% tail DNA]. Thus, for the comet assay it is important to store cells in a way that avoids DNA damage, which may or may not be associated with high viability of cells.

The purpose of this paper is to summarise the literature on the effect of cryopreservation of white blood cells and describe protocols for isolation, cryopreservation and thawing of peripheral blood mononuclear cells (PBMCs), buffy coat (BC) and whole blood (WB) for the comet assay. BC is a mixture of leukocytes and platelets, whereas PBMCs are isolated mononuclear cells. In order to put the experimental work on the comet assay into a meaningful context, we describe briefly the principles of cryopreservation of cultured cells in a historic perspective. The paper encompasses applications of the comet assay to measure DNA strand breaks, enzyme-sensitive sites, and cellular and *in vitro* DNA repair activities. The enzyme-sensitive sites include oxidatively damaged DNA measured by specific recognition of these lesions using bacterial formamidopyrimidine DNA glycosylase (Fpg) or

human oxoguanine DNA glycosylase (hOGG1). The cellular repair assay (or challenge-comet assay) determines removal over time of DNA lesions previously induced in cells. The *in vitro* repair assay measures the activity of cellular extracts on substrate DNA with lesions specifically detected by either base excision repair or nucleotide excision repair.

Principles of cryopreservation of cells

The main problem in cryopreservation is the intracellular formation of ice crystals, which may cause mechanical damage to the cells or secondary effects related to changes of the solutes in the liquid phase of the sample [7]. The experimental work on cryopreservation of cells started approximately 70 years ago with the introduction of glycerol to avoid the formation of intra-cellular ice crystals at a storage temperature of -80°C [8]. Dimethyl sulfoxide (DMSO) was originally introduced as an alternative anti-freezing agent that had low toxicity and higher permeability to cells than glycerol [9]. The use of serum in freezing medium was early on noted to have a beneficial effect on the survival of cells upon thawing and re-establishment of a cell culture [10]. This beneficial effect of serum might be related to avoidance of osmotic shock during thawing of cells. Studies in the late 1960s indicated that cryopreserved cells in medium with serum and anti-freeze agent were viable after 4.5 years of storage [11]. Other fundamental issues noted early on were the superior effect of slow cooling rate (approximately -1°C/min) and fast warming rate for the viability of the cryopreserved cells [12;13]. By now, cell repositories have had cell lines stored for decades, as for instance the monocytic THP-1 cell line in the American Type Culture Collection (ATCC) repository, which was isolated from a patient with acute monocytic leukaemia about four decades ago [14]. The stock in the ATCC repository, cryopreserved in complete cell culture medium with 10% foetal bovine serum (FBS) and 5% DMSO is still viable after four decades. In fact, this particular cell line has been used in comet assay ring-trials to assess and

reduce variation in comet assay procedures between laboratories because it has a low background level of DNA migration in the comet assay [15-17]. THP-1 cells are also recommended for establishment of cryopreserved positive assay controls for the enzyme-modified comet assay [18].

The comet assay has been used for approximately 30 years in human biomonitoring studies on effects of host factors, exposures and diseases [2;19]. The knowledge base of effective storage of cell lines was already present when the comet assay was implemented in molecular epidemiology studies. This is clearly seen in the freezing medium that has been used for storage of PBMCs (often referred to as "lymphocytes"). Cryopreservation of PBMCs for the comet assay has been successfully carried out in medium with 10% DMSO and serum (from 10% to 90%) with storage at -70°C or colder [20-32]. The large variation in serum is due to the use of cell culture medium in the freezing solution. It seems that some researchers use the same full medium as they would have used for the culture of PBMCs (with an addition of 10% DMSO), whereas others increase the content of serum at the expense of cell culture medium. WB and BC have typically been processed as fresh samples in the comet assay.

Storage of suitable material in biobanks

Biobanks are repositories of cryopreserved tissues, cells or non-cellular biological matrices. Research biobanks have focussed on samples from which DNA polymorphisms, gene expression, and proteins can be analysed as well as soluble molecules in plasma, serum or urine [33]. Preservation of viable cells makes it desirable for DNA analyses such as polymorphisms because stimulating of cryopreserved cells to proliferate can markedly increase the DNA material [34]. DNA damage analysis of samples from biobanks established in large-scale, long-term human biomonitoring studies could provide valuable information on the relationships between diet, nutritional status, lifestyle and environmental factors, and the

incidence of cancer and other chronic diseases. Types of biobank samples can be found in the directory of the European Research Infrastructure for Biobanking (BBMRI-ERIC) (http://www.bbmri-eric.eu/). However, as most cohort studies with biobanks require a rather large number of subjects and long-term follow-up to have sufficient statistical power, there are important constrains to consider when storing biological material [2]. Existing biobanks have been forced to make compromises in terms of the type of samples that are collected for cryopreservation. The European Prospective Investigation into Cancer and Nutrition (EPIC) study branch in Heidelberg, Germany, has cryopreserved PBMCs in their biobank from the samples that were collected at re-invitation dates, but the original collection consists of BC samples. Very few biobanks have cryopreserved PBMCs in medium that is suitable for analysis of DNA damage by the comet assay. However, a number of biobanks have frozen BC or WB samples in quantities that are applicable for analysis of DNA damage by the comet assay [2]. The *in vitro* DNA repair assay requires large numbers of cells, in excess of the amounts of WB and BC stored in most biobanks.

Collection of blood, bench time and shipping

It is still uncertain if differences in the collection of venous blood affect levels of DNA damage or repair activity in blood cells [2;4]. Heparin, EDTA and citrate are used as anticoagulants. The choice of anticoagulant is most likely determined by methodological issues related to techniques other than the comet assay. For instance, heparin is a concern because it may inhibit PCR reactions [35;36]. Ideally, the blood samples are processed immediately after withdrawal from the vein. However, this may be delayed for several reasons, ranging from transport of samples from the collection site to the laboratory in field studies to the desire to handle samples as a single batch in the laboratory. This period of delay is called bench time and samples may be kept on ice, in a fridge (4°C) or at ambient temperature. It is most likely that ambient or room temperature differs between studies, due

to differences in the location of the laboratory and time of the year. A recent review concluded that the published literature shows inconsistent results of the impact of bench time and temperature on levels of DNA damage in the comet assay and recommended that blood samples should be processed within 4 h after isolation irrespective of the temperature being below 4°C or ambient [2]. The bench time at room temperature may be increased to 6 h, as demonstrated in a recent paper showing that levels of DNA strand breaks and Fpg-sensitive sites were not affected [29]. As protein is relatively more stable, it is likely that a short bench time, which does not affect the level of DNA damage, will not affect the measurement of DNA repair activity by the comet assay.

The impact of blood collection, bench time and cryopreservation can be illustrated by unpublished results from Stopper and Bankoglu (**Figures 1 and 2**). Random samples of PBMCs from a study involving normal and overweight subjects were cryopreserved at -80°C, using Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS, 1% L-glutamine and 10% DMSO. Subsequently, the samples were thawed and processed immediately in the comet assay or incubated at 37°C for analysis of DNA strand breaks. The mean level of DNA strand breaks increases at 1 h and 2 h incubation, whereas there are similar levels of DNA strand breaks directly after thawing and at 16 h of incubation **(Figure 1)**. Interestingly, the cell number decreased by 25% (standard deviation = 14%) at 16 h as compared to the cell number at the start of the incubation period. **Figure 2** depicts the distribution of DNA strand breaks in the cells. As it can be seen, the increase in DNA migration after 1 and 2 h is due to a small number of highly damaged cells. For instance, 4%, 16%, 29% and 0% of the comets have more than 30% tail DNA at 0, 1, 2 and 16 h incubation, respectively. After 16 h, the numbers of comets in the categories with smaller damage remain the same, but the cells in the higher damage categories have disappeared.

This yields an overall smaller percentage tail DNA. This might be due to disappearance of dead or dying cells rather than repair of DNA strand breaks.

Certain studies may entail shipping of cryopreserved samples between laboratories. This may affect the level of DNA damage, although it is a topic that has not been thoroughly investigated. **Figure 3** depicts the results from a previously unpublished experiment where PBMCs from a human subject were cryopreserved at -80°C for 16 h, using Minimal Essential Medium (MEM) with 10% DMSO, 5% antibiotics and 10% FBS, before it was kept on dry ice for 0-72 h. The cryopreservation slightly increased the DNA migration (0 h versus fresh samples), and the subsequent storage on dry ice had a minor effect too. However, the strongest effect was observed in samples that had been cryopreserved at -80°C for 16 h, then kept on dry ice for 48 h (representing a shipping condition) and subsequently stored at -80°C for one week.

The examples of previously unpublished results illustrate that DNA damage may occur during the freezing and thawing process, or shipment of samples. It should be emphasized that the examples stem from preliminary experiments where effects of the processing of samples have been assessed. Thus, they have been used to develop procedures with minimal generation of DNA damage such as the specific protocols that are described later in the article.

Stability of DNA damage in cryopreserved cells, buffy coat and whole blood samples

We have assessed the effect of cryopreservation in previously published papers as well as unpublished results. The articles were identified by a step-by-step search in PubMed as follows: 1) “Comet Assay” [12333 hits], 2) search “1” AND (Cryopreservation OR Freezing OR Thawing)” [204 hits], and 3) search “2” AND (Blood OR Leukocytes OR Lymphocytes OR Mononuclear Cells OR PBMCs)” [65 hits]. The Boolean operators “AND” and “OR”

were used to combine searches or search terms. The titles and abstracts were assessed to consider the articles for inclusion in the review. In addition, auxiliary searches were done on Google to find publications that are not indexed in PubMed. This yielded two articles that are included in the review [37;38]. We have been particularly interested in finding publications that have reported findings on the stability of cryopreserved samples. We suspect that many researchers may not have considered it important to report that samples are stable upon cryopreservation, whereas demonstrations of an effect of cryopreservation are easier to find.

In the review, we report original results in primary comet assay descriptors as used by the authors. Levels of DNA damage are mainly reported as % tail DNA and visual score. The latter is based on a classification system where comets are scored in five different classes, depending on the DNA migration. It is custom to score 100 comets per sample, yielding a total score between zero and 400 arbitrary units (a.u.). The % tail DNA has a range from zero to 100%. There is a linear relationship between % tail DNA and visual score. In the present review, the visual score divided by four is equivalent to % tail DNA. A few studies in the review have used tail moment as primary comet assay descriptor. The tail moment is not recommended as primary comet assay descriptor because it is difficult to interpret the values in terms of DNA migration. In these cases, we transformed the tail moment units to % tail DNA [25], or compare tail moments in the samples with levels in human PBMCs in the same article [39].

Levels of % tail DNA or visual score in arbitrary units might be difficult to understand unless you are familiar with the comet assay. Transformation of primary comet assay descriptors to more general descriptors, such as number of lesions per unaltered base pairs, has not been standard practice in most articles on comet assay results [40]. In perspective, the values reported in the review can be compared to levels that are typically reported in human PBMCs or leukocytes. Based on a literature review, the % tail DNA reference values in human blood

samples are 8.6% (inter-quartile difference = 10%) and 7.6% (inter-quartile difference = 10%) for DNA strand breaks and Fpg-sensitive sites, respectively [41]. These are similar to levels of DNA strand breaks (9.0% tail DNA) and Fpg-sensitive sites (9.3% tail DNA) in cryopreserved PBMCs, which were analysed in 13 different laboratories in the European Comet Assay Validation Group (ECVAG) ring trial [42].

Observations from cryopreserved assay controls and cell lines

A number of studies have used cryopreserved samples of cells as reference controls, quality controls, positive controls or other terms to control for experimental variation in the comet assay (**Table 2**). To the best of our knowledge, cryopreserved assay controls were first used in a long-term biomonitoring study with repeated sampling of blood from the same subjects throughout approximately 15 months in 1997 and 1998 [39]. The authors used cryopreserved samples of lymphoma cells to assess the stability of the comet assay. The samples were prepared in such a way that a vial could be retrieved from the freezer and directly embedded in agarose without centrifugation and resuspension in fresh medium. Freshly isolated PBMCs from the study subjects, together with cryopreserved assay control samples, were analysed once a week for 62 weeks (results on assay controls are shown in **Figure 4**). The temporal variation in assay controls was not reported in the original publication, although it was mentioned that there was no systematic seasonal variation or trend over time [39]. The main reason for not reporting the results from the cryopreserved assay controls was that it was considered to be well-established that DNA strand break levels were stable in cryopreserved cells (Møller et al., personal communication). This may be an example of reporting bias on the stability of DNA damage levels in cryopreserved cells. Researchers from the same laboratory later published an extensive assessment of results on assay controls, which had been analysed systematically over a 10-year period [43]. Each batch of assay controls typically lasted one year and the statistical analysis of the dataset did not indicate an

accumulation of DNA strand breaks over these 1-year periods in cryopreserved lung epithelial A549 cells [43]. Other authors have also shown that DNA strand break levels in cryopreserved lung epithelial A549 cells and glioblastoma cells were stable for eight weeks when the samples were stored at -80°C, using 90% FBS and either 10% DMSO or 10% cell culture medium [44]. Another study showed that cryopreservation of lymphoblastoid cells did not affect the level of DNA strand breaks, although the storage period was not specified [23].

Samples from the ECVAG ring-trial may shed some light on the effect of long-term cryopreservation. The first ECVAG ring-trial used cryopreserved THP-1 cells as calibration curve samples in order to standardize DNA damage levels between laboratories [45]. Calibration curve samples are used to transform the primary comet assay descriptor such as % tail DNA into the actual number of lesions per number of nucleotides or base pairs, using the knowledge that low linear energy transfer ionizing radiation yields a specific number of DNA strand breaks (see Møller et al. [46]). The participating laboratories in the ECVAG ring-trial received samples of cryopreserved THP-1 cells that had been exposed to ionizing radiation (0, 2.5, 5 and 10 Gy) before cryopreservation at -80°C in RPMI 1640 medium with 50% FBS and 10% DMSO [15]. In addition, the first ECVAG ring-trial also tested the variation in comet scoring on pre-made slides of comets from the calibration curve samples, which were made in the central laboratory and distributed to the participating laboratories. **Figure 5** shows some results from these calibration curve samples. The "Original (2007)" results are the mean level of DNA strand breaks in the laboratories that used an image analysis system from Perceptive Instruments to score the premade-slides in (or around) 2007. At that time, the central laboratory did not have this particular image analysis system. However, "Old (2020)" corresponds to the same calibration curve samples from 2007 analysed using the same comet assay protocol in the central laboratory and scored using the Perceptive Instruments image analysis system in 2020. In addition, a new preparation of

calibration curve samples was carried out by the central laboratory in 2020, using the same cryopreservation procedure, comet assay protocol and image analysis software ("New (2020)"). Collectively, the results in **Figure 5** indicate that the cryopreservation technique used in the ECVAG ring-trial is sufficiently robust to avoid accumulation of DNA strand breaks during a 13-years storage period at -80°C. The cryopreservation was relatively simple as samples were placed in the freezer after the cells had been irradiated, centrifuged and re-suspended in cryopreservation medium. A more important feature might be that the samples were prepared in a volume that makes it possible to directly process the samples in the comet assay. For example, 75 µL cell suspensions can be mixed with 600 µL agarose and applied as four gels (120 µL aliquots on area of 3.85 cm^2 Gelbond) for the comet assay. Cryopreserved cells can be mixed directly with agarose and processed in the comet assay without washing and centrifugation steps.

The effect of cryopreservation on the enzyme-modified comet assay has not been assessed to the same extent as DNA strand breaks. **Figure 6** depicts previously unpublished results from a study on the stability of Fpg-sensitive sites in HeLa cells that were exposed to the photosensitizer Ro19-8022 and irradiated with UV-light before cryopreservation in Dulbecco's Minimal Medium (DMEM) with 20% FBS, 5% antibiotics and 10% DMSO at -80°C (Azqueta et al. previously unpublished results). This treatment produces Fpg-sensitive sites and little concomitant generation of DNA strand breaks. Storage up to 20 weeks did not affect the level of Fpg-sensitive sites. However, it is clear that experiments are warranted on longer periods of cryopreservation on samples that are used for the enzyme-modified comet assay on oxidatively damaged DNA as well as alkylation damage and other types of lesions that can be detected by enzymes in the comet assay.

The scoring of slides of assay control samples is typically done right after the comet assay experiment. Thus, a drift in the level of DNA damage in assay controls could in theory be due to a difference in the intensity of fluorescence in the microscope. Moreover, the scoring may occur over a very long time where new microscope lamps replace lamps that have burned out. The microscope lamp intensity has been hypothesized to affect the detection of comets, although there are not systematic studies published on this type of experimental bias due to technical issues. Interestingly, Bankoglu et al. have done an experiment resulting from a study with patients before and one-year after bariatric surgery, where assay controls were used to control for variation over time [47]. HL-60 cells were treated with DMSO or methyl methanesulfonate (MMS) and cryopreserved. After treatment, cells were diluted in freezing medium (RPMI 1640 containing 10% FBS, 1% L-glutamine and 10% DMSO) with a density of 1×10^6 /ml and aliquoted in 250 µL volumes. These aliquots were frozen in a freezing container with isopropanol at -80°C overnight and stored at -80°C. On the day of experiment, one aliquot from each negative and positive control were thawed rapidly and mixed with low melting point agarose. Extra slides were prepared, but not analysed in the original study. In connection to the present review, Bankoglu et al. have stained and scored samples on the same day (**Figure 7**). The results do not suggest an effect of cryopreservation on levels of DNA strand breaks in unexposed or MMS-exposed cells.

Effect of cryopreservation in PBMCs

The effect of cryopreservation has received irregular attention in the context of biomonitoring studies (**Table 3**), and the stability of DNA damage levels during long-term storage has only been investigated in recent years. Early studies demonstrated no immediate effect of cryopreservation of isolated lymphocytes that were frozen to -70°C in medium with 20% plasma and 10% DMSO [20]. This was only based on samples from three subjects and 4 h of

storage in the freezer. However, the authors highlighted that improper cryopreservation increased the background level of DNA strand breaks [20]. At the same time, it was reported that the level of DNA strand breaks in cryopreserved PBMCs (10% DMSO in FBS at -70°C) was not different from fresh samples when assessed 1-3 days after freezing, whereas higher levels of DNA strand breaks were observed in samples after a longer time of storage [48]. A more systematic study showed that fresh and cryopreserved PBMCs at -70°C, using 50% FBS, 40% RPMI 1640 medium and 10% DMSO had the same level of DNA strand breaks and sensitivity to additional breaks caused by *ex vivo* treatment with H_2O_2 and γ-radiation [21]. Unfortunately, the period of cryopreservation was not reported, although the authors highlighted that it was possible to store PBMCs for long periods. The same goes for a study where cryopreservation of PBMCs for an unspecified period of time did not affect the level of DNA strand breaks [22].

A number of studies have assessed stability of the levels of DNA strand breaks and Fpg-sensitive sites in PBMCs, using various combinations of cell culture medium with FBS and 10% DMSO. No statistically significant accumulation of DNA strand breaks or Fpg-sensitive sites has been observed in a number of studies on PBMCs that have been cryopreserved up to one year at -80°C in cell culture medium with FBS and 10% DMSO [24;26;28-31]. There is one study that assessed the effect of freezing of blood cells that were isolated by Histopaque 1077 (regarded as PBMCs, although referred to as "leukocytes" by the authors) [25]. The authors reported an increased level of DNA strand breaks in frozen samples (3.3 versus 0.4 tail moment units). The tail moment is renowned for being unsuitable for inter-laboratory comparisons. However, the authors also reported that the maximum level of DNA migration was 198 tail moment units [25]. Assuming a linear relationship between tail moment units and % tail DNA (and the maximal DNA migration is close to 100% tail DNA), it can be speculated that the net difference in tail moment units (i.e. 2.9) corresponds to less than

≈1.5% tail DNA or a very modest increase in DNA migration despite the statistically significant effect. Another study showed a relatively strong effect of cryopreservation on levels of both DNA strand breaks (increase from 6.3% to 11.7% tail DNA) and Fpg-sensitive sites (increase from 11.5% to 21.2% tail DNA) in PBMCs that had been stored in RPMI 1640 medium with 50% FBS and 10% DMSO [27]. A similar effect on levels of DNA strand breaks and Fpg-sensitive sites has recently been reported by the same researchers, using cryopreservation at -80°C for 4-12 month [32].

Figure 8 shows the net difference in DNA strand break levels in PBMCs after storage at -80°C compared with freshly isolated cells in studies that have used % tail DNA as primary comet assay descriptor [24;27;29;30]. It suggests that a slightly increased level of DNA strand breaks is observed in cryopreserved PBMCs, but it does not indicate that DNA damage accumulates over one-year storage at -80°C. The effect of longer than one-year storage on DNA strand break levels in PBMCs has not been assessed in a systematic way. However, there exists one study where PBMCs were used as "external quality control samples" in a clinical trial [49]. PBMCs were exposed to ionizing radiation (20 Gy) and subsequently cryopreserved in Hanks Balanced Salt Solution with 10% DMSO. Thawed samples that were analysed over 30-months period did not indicate any accumulation of DNA strand breaks (**Figure 9**). **Figure 10** shows previously unpublished results on cryopreserved PBMCs using the same protocol as a validation on a high through-put version of the comet assay [50]. The samples were used at weeks 8-88 after cryopreservation. The researchers used Trevigen slides in weeks 8-40 and Gelbonds in weeks 44-88 (depicted as two different strata of data in **Figure 10**). The extended cryopreservation had no effect on DNA strand break levels. Unfortunately, there was not an assessment of DNA strand break levels in fresh samples, but the reported levels of DNA strand breaks (i.e. approximately 3% tail DNA) is relatively low.

Effect of cryopreservation of whole blood

The use of WB has several advantages compared with isolated cells, especially for longitudinal human biomonitoring studies in which typically large sets of samples have to be processed at once [29;51;52]. Using WB takes substantially less time compared with the isolation of PBMCs. Besides, one needs only a few microliters of WB for the analysis. This is important in biomonitoring studies, where available volumes are often restricted due to ethical considerations or the need to conserve stocks for various assays and measurements. Another advantage of using WB is the avoidance of potential additional DNA damage occurring as a consequence of cell isolation.

A number of comet assay studies have emerged in the last decade on cells from WB (**Table 4**). Anderson and co-workers experimented with storage of blood samples at -20°C without cryopreservative, but these storage conditions generated high levels of DNA strand breaks [53]. DNA damage can be measured in small volumes of WB in freezing medium [38;54;55] or without cryopreservatives [26;30]. A "small volume" refers to 200-400 µL blood. In comparison, a large volume of blood refers to several millilitres that are typically obtained in standard heparin or EDTA tubes for isolation of PBMCs, although PBMCs can also be isolated from small volumes of blood [39;56]. As depicted in **Figure 8**, the majority of studies have shown that WB samples have been stored up to one year show no sign of increased levels of DNA strand breaks [28-30;37;54;57;58]. An exception is a recent study that showed higher levels of DNA strand breaks in cryopreserved WB after storage for 4-12 months as compared to freshly isolated WB from the same donor [32]. The analysis includes WB samples with freezing medium [28;29;37;54;57] and samples that have been cryopreserved in small volume without cryopreservative [30;58]. Similar results have been obtained for the Fpg-modified comet assay [28-30].

Leukocytes can be isolated from cryopreserved WB, by a simple centrifugation and washing procedure. These isolated cells have the advantage over WB that they can be challenged with H_2O_2 to estimate the antioxidant status of the cells – a method established with PBMCs and used in various nutritional supplementation trials. WB is unsuitable, as catalase and/or haem from red blood cells (RBCs) break down the H_2O_2. The levels of basal DNA damage in a set of nine samples from human volunteers were higher in isolated leukocytes from cryopreserved WB than in cryopreserved WB or PBMCs, indicating some strand breakage during isolation. However, there was a high correlation between H_2O_2-induced breaks and Fpg-sensitive sites (both reflecting antioxidant status), implying that such measurements have biological significance [26].

A recent study showed slightly higher level of DNA strand breaks in WB that had been stored for five years compared with freshly isolated samples, although it should be noted that the cryopreserved and fresh samples were not obtained from the same subjects [59;60]. Nevertheless, both fresh and frozen WB samples had low levels of DNA strand breaks (i.e. 4.4% and 1.6% tail DNA). Al-Samani et al. found an increase in damage after long-term storage of larger volumes (at about 5 ml), but not small volumes of WB, indicating the importance of cryopreserving WB in small aliquots (e.g. 250 µL) or less [55].

As it currently stands, there are two ways of cryopreserving WB that give reliable results in the comet assay: 1) snap freezing of blood in small volume (i.e. 200-400 µL) in liquid nitrogen or in a freezer at -80°C, 2) mixing large volume of blood with freezing medium and slow-freeze in a similar way as the cryopreservation of PBMCs.

Effect of cryopreservation of buffy coat samples

After low-speed centrifugation of anticoagulated blood, the upper part of the RBC pellet is enriched in platelets and leukocytes which give it a buff tinge - hence the name of 'buffy coat'. It is mainly used to prepare human donor leukocytes and platelets, to prepare DNA from blood, or to differentiate leukocytes in a smear. The employed speed of centrifugation is not the same in all protocols, and some authors refer to BC for density-gradient isolated fractions. Thus, the composition of this fraction can vary among different laboratories and publications.

Recent publications have indicated that the comet assay can be performed on cells from cryopreserved BC (**Table 5**). The work of Ladeira et al. showed a progressive increase in strand breaks and oxidative damage in BC samples preserved in cryostraws up to 12 weeks [30]. However, it is possible that the increased damage was associated with the thawing procedure, which involved pushing the frozen BC out of the cryostraw. Thus, future work will need to clarify some important points, such as the relation between BC preparation and composition, differences between freezing in cryovials or cryostraws, and thawing procedure.

Furthermore, leukocytes can be isolated from cryopreserved BC, by a similar centrifugation and washing procedure to that described above for WB. DNA strand breaks in BC and BC leukocytes were higher than in fresh PBMCs, but still relatively low, with a median % tail DNA of around 2%. High correlations were seen between BC leukocytes and PBMCs, for both DNA strand breaks and Fpg-sensitive sites [61].

Stability of DNA repair activity in cryopreserved cells, buffy coat and whole blood samples

Comet-based repair assays provide quantitative and functional determination of an individual's repair response capacity to induced DNA damage. There are two different variants for this purpose: the challenge-comet assay (or cellular repair assay) and the *in vitro* repair assay.

The use of cryopreserved samples in the challenge-comet assay

The basic principle of the challenge-comet assay is to treat (challenge) cells with a genotoxic agent and then to determine how cells respond to the challenge in terms of repairing the genetic damage induced by measuring the remaining damage over time with the comet assay [62]. A major advantage of this technique is that it allows to collect data at multiple time points after the challenge, thereby determining the kinetics of repair activities, and to evaluate alterations in a variety of DNA repair pathways, or even in different steps of the same pathway, by employing specific genotoxicants as challenging agents [21;63] and/or different DNA repair inhibitors [64].

The challenge-comet assay has been widely used for measuring the repair activity in a number of epidemiological studies employing both fresh and cryopreserved samples, primarily isolated PBMCs. In particular, using frozen samples, the challenge-comet assay was employed to evaluate the effects on individuals´ DNA repair capacity of environmental exposures - including arsenic [65], seasonal solar exposure [66], smoking [67], and environmental pollution [68], of an exercise intervention in a population of overweight women [69], of radiotherapy in breast cancer patients [70], of frailty status in older adults [71], and of different features, such as demographic - age, sex, and ethnic group [23] - or clinical features - non-small cell lung cancer [72]. PBMCs were used in all these studies;

however, different challenge agents (including γ-radiation, H_2O_2, 2-acetoxyacetylaminofluorene, UV, and bleomycin), and treatment conditions (with or without prior stimulation with phytohaemagglutinin (PHA), and different treatment times and repair periods), together with different comet assay protocols, were employed.

Several studies have addressed the suitability of frozen samples to be employed in the challenge-comet assay. Most of these works compared the use of fresh or cryopreserved isolated human PBMCs [21-23;73;74]. The first study conducted reported that the repair capacity of cryopreserved PBMCs exposed to γ-rays and H_2O_2 was the same as that of the fresh counterparts, in the presence of PHA only during the repair period [21]. Besides, Allione et al. also found significant correlation between results obtained from employing fresh or cryopreserved PHA-stimulated PBMCs to evaluate benzo[a]pyrene-diol-epoxide induced DNA damage repair capacity by using the challenge-comet assay in combination with the nucleotide excision repair (NER) inhibitor aphidicolin [73]. Using the same assay variant, frozen PBMCs were reported to behave similarly to fresh PBMCs, but after a 16-h recovery period in the presence or in the absence of PHA (Stopper and Bankoglu, personal communication).

Three independent studies tested the ability of the challenge-comet assay to effectively measure DNA repair capacity in cryopreserved PBMCs without prior culture or PHA stimulation. Firstly, Duthie et al. reported that cryopreservation reduces the capacity for DNA excision repair in non-mitogen-stimulated frozen PBMCs challenged with H_2O_2 [22]. However, Chang et al., using γ-irradiation as challenge agent, demonstrated that results obtained from cryopreserved PBMCs employed immediately after thawing, without cell culture or other extensive manipulation, were correlated with those obtained from fresh samples; though, on average, the DNA repair capacity was 14% lower in cryopreserved

samples [74]. Accordingly, Trzeciak et al. also reported no difference and good correlation in DNA repair capacity between fresh and cryopreserved unstimulated PBMCs challenged with γ-irradiation [23].

Finally, a recent work demonstrated the suitability of WB samples, both fresh and frozen, as an alternative proper biomatrix for the challenge-comet assay after a 24 h incubation in presence of PHA. Both sample types were equally efficient, and comparable in activity to cryopreserved PHA-stimulated PBMCs, in evaluating the capacities in the key NER, base excision repair (BER) or double strand break repair pathways [75].

The use of cryopreserved samples in the in vitro repair assay

The *in vitro* DNA repair assay is based on the ability of protein extracts, from cells or tissues, to recognise and incise in the sites of the specific DNA lesions artificially induced in substrate cells [76;77]. This version of the comet assay can be used to assess BER or NER, depending on the type of lesions present in the substrate cells (i.e. DNA oxidation damage, or UV-photoproducts or benzo[a]pyrene-diol-epoxide induced DNA adducts, respectively). This assay has been applied successfully to assess the effect of extrinsic and intrinsic factors on an individual's DNA repair activity in biomonitoring studies, occupational exposure assessment, nutritional interventions, animal studies, as well as clinical investigations [1;78]. For human biomonitoring studies, mainly PBMC extracts have been used to assess the individual DNA repair activity [1;78].

The assay is rarely performed in fresh samples; normally PBMCs are isolated from the volunteers and slowly frozen in freezing medium or snap-frozen as cell pellet. In any of the cases, the samples are stored at -80ºC until the day of the analysis, when the extraction procedure is carried out [77]. The assay cannot be performed with WB due to the presence of haemoglobin. It is worth taking into account the relatively large number of cells required for

this assay (at least 2×10^6 cells to ensure proper protein extraction), which may not be possible to isolate from small aliquots of frozen WB. Applying RBC lysis to isolate leukocyte pellets from fresh WB might be logistically more attractive and less time-consuming than PBMC isolation in biomonitoring studies to assess DNA repair activity. So far, the use of RBC lysis to prepare leukocyte extracts has not been tested in the *in vitro* DNA repair assay, since there are contradictive reports on the effect of RBC lysis on DNA damage levels and enzyme activity [61;79]. Gafter-Gvili et al. reported RBC and RBC hemolysate to have a protective effect, rather than a damaging effect, on DNA damage levels and DNA repair [79]. The use of BC for the determination of the DNA repair activity has not been tested either. However, leukocytes could be isolated from frozen BC; an aliquot of 250 µL should yield ~2 $\times 10^6$ leukocytes ([30] and https://www.cptp.inserm.fr/wp-content/uploads/2018/01/PBMC-isolation-and-cryopreservation.pdf).

In this assay, the cryopreservation is focused on the preservation of the DNA repair enzymes activity. In this regard, stability studies were performed, but only few using human blood cells have been published. Langie et al. [80] showed that PBMC pellets stored for up to 40 days could be used to prepare cell extracts and study NER without losing enzyme activity (for one person pellets were tested at 4.5 months, showing similar activity – unpublished data), while long-term storage of the prepared cell extracts at -80ºC requires the addition of ATP to restore enzyme activity [80]. However, to assess BER activity the enzyme activity is generally preserved at -80ºC, as shown for mice tissues (up to 18 months at -80 ºC) and corresponding extracts (up to 10 months -80 ºC) [81]. Other DNA repair assays based on the same principle (i.e., the cell extract nicking activity towards a plasmid or nucleotides containing certain lesions) also used frozen samples: Millau et al. used frozen PBMCs (in freezing medium containing RPMI 1640 medium, 10% DMSO and 10% FBS) as cell suspension and stored in liquid nitrogen, for the further extract preparation and analysis using

a damaged plasmid microarray [82]. Crosbie et al. also used frozen PBMCs (in PBS) to prepare nuclear extract for an oligodeoxynucleotide cleavage assay [83].

Specific protocols on PBMCs, buffy coat and whole blood

There are most likely as many blood collection protocols as there are comet assay laboratories. Below are consensus protocols for the preparation and storage of WB, BC and PBMCs. By "consensus" means that the authors of this review have consented to the feasibility of the protocols, but each author has adapted the protocol to the particular equipment in the laboratory.

From the published literature and experience from the authors of the present review, heparin, EDTA and citrate can be used as anticoagulant for both measurement of DNA damage and repair activity by the comet assay. However, other biomarkers or assays might be flawed by use of specific anticoagulants. Thus, it is recommended to choose blood collection tubes with anticoagulant that do not affect the panel of biomarkers or assays in the study.

Preparation and storage of small volume of whole blood and buffy coat

To store venous blood (collected in a commercial tube with anticoagulant such as heparin, EDTA or citrate):

1) Draw 200-250 µL aliquots of blood in 1.5 mL microcentrifuge tubes
2) Snap-freeze the tubes by placing them on dry ice for a few min
3) Transfer the tubes to a −80°C freezer. [Alternatively, simply place the tubes in the −80°C freezer without snap-freezing on dry ice].

For isolation of BC

1) Draw 200-250 µL aliquots of blood in 1.5 mL microcentrifuge tubes
2) Centrifuge the tubes for 10 min at 400 × *g*, room temperature.
3) Remove the buffy coat in about 0.5 mL and transfer 200-250 µL aliquots to 1.5 mL microcentrifuge tubes.
4) Snap-freeze as above and store at −80 °C.

Isolation of leukocytes from small volume of cryopreserved whole blood or buffy coat

The protocol is based on collection of 250 µL WB. The advised volume contains the appropriate number of cells for one assay, but it also works with high volumes of WB and up-scaling of the volumes in the protocol.

1) Thaw the frozen aliquots of WB or BC quickly by placing in a 37°C water bath.
2) When thawed, immediately place on ice.
3) Mix 100 µL of WB or 50 µL of BC with 1 mL of ice-cold PBS in a microcentrifuge tube
4) Centrifuge for 7 min at 400 × *g*, 4°C.
5) Discard the supernatant (containing lysed red cells)
6) Resuspend the pellet in 1 mL of ice-cold PBS
7) Centrifuge for 7 min at 400 × *g*, 4°C.
8) Repeat this washing procedure twice.
9) Suspend the BC leukocytes in 1 mL of cold PBS.
10) Repeat washes until the solution is no longer red (i.e. it is necessary to remove traces of red cells, haem, etc).

Isolation of peripheral blood mononuclear cells

PBMCs can be isolated by density gradient centrifugation. It should be mentioned that a tutorial video exist for the isolation of PBMCs and comet assay procedure on the International Comet Assay Working Group website (https://youtu.be/tgNHWVqF52I). In brief, the blood collection and PBMC protocol contain steps as follows:

1) Place blood on top of a density gradient medium such as Histopaque, Ficoll-paque or Lymphoprep (1:1 volume ratio) in a test tube. To increase the purity and efficiency of the mononuclear cell isolation, blood samples can be diluted either with PBS or with RPMI 1640 medium containing 2% FBS (1:1) before layering on the density medium.
2) Centrifuge the tube at room temperature 1000 × *g* for 30 min (slow acceleration, no brake).
3) Collect the cloudy layer just above the density medium (containing mononuclear cells) into a 15 mL tube.
4) Dilute the layer containing PBMCs 1:4 with RPMI 1640 medium + 2% FBS.
5) Centrifuge 1000 × *g* at room temperature for 30 min.
6) Discard the supernatant and resuspend the pellet in 0.5 mL cold RPMI 1640 medium + 2% FBS.
7) Determine the cell number (by using e.g. a cell counting chamber).
8) Centrifuge 1000 × *g* at room temperature for 30 min.
9) Resuspend PBMCs in cryopreservation medium to the desired cell density (e.g. 2 × 10^6 cells/mL). The freezing medium should contain 10% DMSO, whereas the other composition of other components is not critical. A typical freezing medium is RPMI 1640 medium containing 10% FBS and 10% DMSO. A freezing medium with 40%

RPMI 1640, 50% FBS and 10% DMSO has been used by a number of the authors on the present paper.

10) Freeze the isolated PBMCs slowly overnight in a freezing container with isopropanol at -80°C (or in a Styrofoam box) and store at -80°C. [Alternatively, for the comet-based *in vitro* DNA repair assay, PBMCs can be stored at -80°C as cell pellets of 2-5 × 10^6 cells/pellet].

Isolation of leukocytes from fresh whole blood

1) Mix blood 1:1 with lysis-buffer (8.29 g NH_4Cl (155 mM), 1.0 g $KHCO_3$ (10 mM), 0.372 g EDTA (1.0 mM), dissolved in 1000 mL H_2O; pH 7.4, sterile filtrated).
2) Lyse erythrocytes. Keep the tube on ice and shake carefully every 5 min. Normally cells lyse within 20-30 min; cells are lysed when suspension gets a clear dark-red colour.
3) Centrifuge cells at 300 × *g*, 5 min at 4 ˚C.
4) Decant red coloured supernatant.
5) Resuspend pellet in lysis buffer (1 vol) and lyse again for about 5 min to remove spare contaminating erythrocytes.
6) Centrifuge cells at 300 × *g*, 5 min, 4 ˚C.
7) Decant supernatant; a clear white pellet containing leukocytes should be visible.
8) For freezing of the leukocytes, follow steps 6-10 as described for PBMCs above.

Remarks

The comet assay can be carried out on both fresh and cryopreserved samples. As demonstrated in this review, the level of DNA strand breaks in PBMCs is relatively stable in samples that have been stored at -80°C for years. We recommend that samples are processed immediately after thawing to avoid the formation of DNA strand breaks. Uncertainty pertains

to the stability of Fpg-sensitive sites and other DNA lesions that are detected by the enzyme-modified comet assay. This knowledge gap is currently being investigated in a study on the stability of potassium bromate as a positive assay control for the Fpg-modified comet assay [84]. Nevertheless, the results indicate that cryopreservation causes only a minor increase in levels of DNA damage in PBMCs. The majority of the studies in the review indicate that the net increase in DNA migration is a few % tail DNA units in cryopreserved PBMCs. This difference in DNA damage is barely visible in the microscope. As a reference, a meta-analysis has shown that the level of DNA strand breaks is approximately 4% and 15% tail DNA in subjects who were categorized into age groups <20 years and >64 years [84]. Likewise, a meta-analysis has indicated that patients with various types of diseases have approximately two-fold higher levels of DNA damage in PBMCs or leukocytes as compared to healthy subjects [6]. In that respect, the effect of cryopreservation is most likely negligible, although it should also be emphasized that some authors have shown evidence of accumulation of DNA damage in cryopreserved samples. This accumulation of DNA damage might be due to specific laboratory procedures such as washing and centrifugation of thawed cells. It should also be noted that these examples are few compared to the number of previously unpublished results in this review. In keeping with the knowledge from storage of cells for a long period of time, it is recommended that the freezing medium contains 10% DMSO, whereas other components are not critical. The majority of researchers use FBS and cell culture medium, but the content of these components differs. There are solid reasons to discontinue the use of FBS in freezing medium because animal welfare concerns and economy (RPMI 1640 medium is cheaper than FBS).

In general, there is a knowledge gap on the effect of cryopreservation of BC for use in the comet assay. Likewise, the use of cryopreserved WB in the comet assay is not as well researched as PBMCs. It appears that cryopreservation of WB does not affect the level of

DNA strand breaks and oxidatively damaged DNA as long as the small volumes of blood is used. However, it is not yet clear why it only works for small volumes of blood. In relation to the comet-based DNA repair assay, the use of protein extracts from total leukocyte fraction has not been reported so far, but should theoretically be feasible. As such, the possibility of isolating leukocytes from cryopreserved BC can make storage of samples to assess DNA repair activity from large-scale studies more attractive; as frozen aliquots of 250 µL can yield a cell pellet of ~2×10^6 leukocytes – enough to prepare a protein extract. The use of protein extracts from leukocytes to assess DNA repair activity is an interesting option that will be investigated in parallel to extracts from PBMC pellets in the near future. Regarding DNA-repair activity, the viability of the thawed cells needs to be taken into account if the procedure involves culturing the thawed cells. Non-viable cells can exhibit large comet assay damage after short terms of culture, but may have disappeared after overnight culture. Both the development and especially the disappearance of such long comets could be misinterpreted as part of a repair process while it really may be caused by the death of heavily damaged cells.

The duration and temperature of the bench time step have been highlighted as conditions, which are not standardized and may affect the level of DNA damage in the comet assay. A number of papers in the review have not specified the bench time and temperature, which may be because the samples were processed immediately after the isolation of blood. It should be noted that reporting that samples were processed “immediately” is imprecise as it is open to interpretation. Articles that have reported precise information, show that bench time and temperature varies from 1 h at room temperature [30], 1 h on ice [28], 3 h at room temperature [23], 4 h at 4°C [59] and 2 h at an unspecified temperature [31]. All of these conditions are within the recommended bench time of 4 h at temperature ranging from less than 4°C to room temperature [2]. It is recommended that bench time and temperature is specified in articles.

In summary, the results from this review of the literature, supported by a number of previously unpublished results to fill certain knowledge gaps, indicate that cryopreserved WB, BC and PBMCs can be used in the comet assay for the measurement of DNA strand breaks, enzyme-sensitive sites and DNA repair activity. Care should be taken when cryopreserving WB, BC or PBMCs. It is recommended that the stability of the samples in freezer or liquid nitrogen is assessed before biomonitoring studies are launched. However, it is not necessary to document the stability over extended periods of storage because it appears that the major effect of cryopreservation occurs within the first couple of days in the freezer.

Conflict of interest

The authors report no conflicts of interest

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Table 1. Pros (advantages) and cons (limitations) of cryopreservation of samples for analysis in the comet assay on DNA damage and repair activity

Samples	Pros (advantages)	Cons (limitations)
Fresh	• No generation of artificial DNA damage. • Subsets of leukocytes can be isolated from blood. • Results can be obtained fast (e.g. within 24 h)	• Inter-individual variation in DNA damage or repair levels may be smaller than the between-day (assay) variation • Logistically not feasible in large (e.g. cohort) studies
Frozen	• Applicable for studies with blood collected outside the lab. • Multiple samples from the same individual can be analysed in the same assay. • Biobank material can be used for retrospective studies. • Conducting long-term epidemiological studies. • Conducting large-scale human biomonitoring studies. • PBMCs/leukocytes can still be isolated from small aliquots of frozen whole blood • Frozen cell pellets are easy to use to assess DNA repair activity	• Generation of spurious genotoxicity by the freezing/thawing procedure • Loss of samples due to freezer malfunction or lack of electrical power • Cryopreservation medium may affect enzyme activity (repair assay) • Cryopreservation medium may mask antioxidant activity • Comparing with data obtained in previous studies using fresh blood samples might be difficult (even when using an assay control). • Frozen whole blood samples cannot be used for the DNA repair assay

Table 2. Effect of cryopreservation and thawing on levels of DNA damage in quality controls (assay controls) or similar samples

Type of cells	Freezing and storage	Thawing	Effect	Reference
L1210 mouse lymphoma cells	40% RPMI 1640, 50% FBS and 10% DMSO. Stored at -80°C for maximally 62 weeks	Heated briefly in the hand and mixed directly with agarose and applied on slides	No effect on levels of DNA strand breaks in 54 samples analysed over a period of 62 weeks (results shown in Figure 4)	[39]
A549 cells (both unexposed and H_2O_2 exposed cells)	80% HAM's F12, 10% FBS and 10% DMSO at -80°C for approximately 12 month	At 37°C and rapidly processed in the comet assay	Reported to be stable over one year. The research group states that they have used these types of assay controls for 10-years (each lasting about one year) and have not observed accumulation of DNA strand breaks as a function of storage time	[43]
Glioblastoma (A172) and lung alveolar epithelial (A549) cells	Cryopreserved at -80°C in FBS with 10% DMSO or cell culture medium with 10% DMSO (cooled first to -20°C for 1 h). Stored for 1, 2, 4 or 8 weeks	Not reported	Overall, no difference in DNA strand break levels between fresh and frozen samples. Some variation between levels of DNA strand breaks in experimental groups have been attributed to differences in the harvesting method (mechanical or enzymatic) and cryopreservation medium	[44]
AG10097 lymphoblastoid cells	40% RPMI 1640, 50% FBS and 10% DMSO. Frozen overnight in isopropanol-containing freezing container at -80°C and then stored at -140°C. Duration of storage not reported	In a 37°C water and processed for gamma-ray irradiation as gel-embedded cells	No difference in background level in fresh or cryopreserved cells (approximately 10% tail DNA in both samples)	[23]
THP-1 cells (exposed to 0, 2.5, 5 or 10 Gy of γ-radiation)	40% RPMI 1640, 50% FBS and 10% DMSO. Stored at -80°C	Heated briefly in the hand and mixed directly with agarose and applied on slides	Unaltered levels of DNA strand breaks in cryopreserved samples that were analysed in 2007 and 2020 (including newly irradiated and cryopreserved cells in 2020). Results shown in	[15]

			Figure 5	
HeLa cells exposed to 1 µM Ro19-8022 and 4 min UV-light	Cryopreserved at -80°C in DMEM with 20% FBS, 5% antibiotics, and 10% DMSO	At 37°C, washed twice in cold PBS (by centrifugation: 5 min, 800 × *g*, 4ºC), resuspended in PBS and then mixed with agarose	Unaltered levels of DNA strand breaks and Fpg-sensitive sites after storage up to 20 weeks (results shown in Figure 6)	NA[a]
HL-60 cells (unexposed and cells treated with methyl methanesulfonate	Cryopreserved at -80°C in RPMI 1640, 10% FBS, 1% L-glutamine and 10% DMSO (frozen overnight in isopropanol freezing container).	At 37°C and mixed with agarose	No difference in unexposed or methylmethane sulfonate samples that had been stored for approximately one year (results shown in Figure 7).	[47]

[a]Not applicable (NA). Unpublished results by Amaya Azqueta (personal communication).

Table 3. Effect of cryopreservation and thawing on levels of DNA damage in peripheral mononuclear blood cells

Collection	Freezing and storage	Thawing	Effect	Reference
Heparin tubes and isolation of PBMCs by Lymphocyte separation medium (3 subjects)	Snap frozen (-70°C) in TC-99 medium, 20% plasma and 10% DMSO. Stored for 4 h	At 37°C (water bath) and processed in the comet assay	No difference in DNA strand breaks between fresh and frozen samples (authors note that improper cryopreservation led to increased levels of DNA strand breaks, suggesting some experimentation to develop a useful cryopreservation protocol)	[20]
Heparin tubes and isolation of PBMCs by Ficoll-Hypaque (17 patients with breast cancer)	10% DMSO in FBS at -70°C (overnight) and -158°C. Storage period not specified	At 37°C and washed with PBS	Multivariate statistical analysis did not indicate an effect of storage. The authors mention that storage for more than 1-3 days can increase the level of DNA damage (results were not shown)	[48]
Heparin tubes and isolation of PBMCs by LymphoPrep (3 subjects)	RPMI 1640, 50% FBS and 10% DMSO at -70°C (-1°C/min). Storage time not specified.	At 37°C. Transferred to thawing medium (50% FBS, 40% RPMI 1640 and 10% dextrose) and centrifuged. Resuspended in cold PBS.	No differences in basal level of DNA strand breaks or additional breaks induced by H_2O_2 or γ-radiation between fresh and frozen samples.	[21]
Blood centrifuged and PBMCs obtained by LymphoPrep (4 subjects). Use of blood collection tubes or anticoagulant are not specified	Stored in 90% FBS and 10% DMSO at -70°C (-1°C/min). Storage period is not specified	At 37°C, then centrifuged and resuspended in RPMI 1640 medium with 10% FBS	No difference between fresh and frozen samples in terms of DNA strand breaks (22 versus 24 a.u.) and Fpg-sensitive sites (57 versus 61 a.u.; 0-400 a.u. scale). *Note*: to put the increase in DNA strand breaks into perspective, one has to imagine that 100 comets are scored. In the fresh samples, 78 comets have a completely round appearance, whereas the 22 comets demonstrate a slight formation of comet tail. In the cryopreserved cells, there will be two extra cells that have	[22]

			formed a slight comet appearance	
Heparin tubes and isolation of PBMCs by Histopaque (3 subjects)	Frozen slowly to -80°C (90% FBS and 10% DMSO). Store for 2-3 days or 4 weeks	Washed/centrifuged twice before processing in the comet assay	Unaltered levels of DNA strand breaks in fresh and 2-3 days, and 4-weeks cryopreserved samples (3.9%, 4.3% and 4.5% tail DNA). Levels of Fpg-sensitive sites were not different between fresh and cryopreserved cells for 2-3 days or 4-weeks (7.2%, 8.8% and 8.4% tail DNA respectively)	[24]
EDTA tubes from 10 subjects. PBMCs were isolated using LymphoPrep). Time from blood collection to isolation of PBMCs is not specified	Stored at -20 or -80°C in DMEM medium with 20% FBS and 10% DMSO (frozen slowly). Storage time not reported	Addition of PBS to PBMCs and centrifugation	Lower levels of DNA strand breaks in cryopreserved cells at -20°C (1.8% tail DNA) and -80°C (0.5% tail DNA) compared to fresh cells (3.4% tail DNA)	[26]
EDTA tubes and placed on ice for maximally 1 h. PBMCs isolate by Histopaque 1077 from 4 subjects	RPMI 1640 medium with 10% FBS, 10% DMSO and 1 mM deferrosamine. Stored for 1, 7, 14 or 28 days at -80°C (1°C per min)	Directly mixed with agarose (thawing procedure not specified)	No difference in DNA strand breaks (tail moment < 1) between fresh and frozen samples. Determination of oxidatively damaged DNA, using the Fpg-modified comet assay, indicate no effect of storage at -80°C. *Note:* quality of the experiments cannot be assessed because there is no positive control for the Fpg-modified assay and the tail moment values give little information about the extent of DNA migration	[28]
Heparin tubes from 30 subjects. Processed within 2 h after isolation of blood (kept at room temperature during bench time)	Frozen in -70% RPMI 1640 medium, 20% FBS and 10% DMSO to -80°C in Mr Frosty Nalgene boxes and stored 12 month.	In a 37°C water bath	Lower levels of DNA strand breaks in frozen PBMCs than in fresh whole blood (3.3 versus 4.0% tail DNA). Lower levels of Fpg-sensitive sites in frozen PBMCs (16.7% tail DNA) than fresh whole blood samples (18.2% tail DNA). Note: the study	[29]

			did not include a true control for PBMCs, so the DNA damage level in fresh whole blood has been used as reference value	
Blood collected from 3 subjects in EDTA tubes and kept maximally 1 h at room temperature before processing	All samples frozen at -80°C (PBMCs in RPMI 1640 medium with 50% FBS and 10% DMSO). Stored for 1, 4 or 12 weeks	At 37°C, then mixed with agarose	Statistically significant increase in DNA strand breaks after 1 (4.5% tail DNA) and 4 weeks (6.0% tail DNA) as compared to fresh samples (1.4% tail DNA; mean level of DNA strand breaks in 12-weeks samples was 5.1% tail DNA). No difference in levels of Fpg-sensitive sites (9.9, 10.6, 16.8 and 12.0% tail DNA) in fresh samples as compared to cryopreservation for 1, 4 or 12 weeks. *Note*: overall the mean levels of DNA damage in cryopreserved samples (i.e. average of 1, 4 and 12 weeks) indicate a slightly higher level than fresh samples (strand breaks: 5.2%versus 1.4% tail DNA, Fpg-sensitive sites: 13.1% versus 9.9% tail DNA)	[30]
Heparin tubes and isolation of PBMC by LymphoPrep (18 subjects). Time from blood collection of isolation of PBMCs did not exceed 2 h (temperature during bench time is not reported)	80% RPMI 1640 medium, 10% FBS and 10% DMSO to -80°C in Mr Frosty Nalgene boxes and then transferred to liquid nitrogen. Stored for one week up to one year (not further specified)	37°C water bath. Centrifuged and resuspended in fresh cell culture medium	Authors highlight that fresh and cryopreserved PBMCs from the same donor had similar levels of DNA strand breaks (DNA strand break levels are less than ≈3% tail DNA). The results on background levels of DNA damage are not specifically reported as the study focussed on DNA repair activity	[31]
EDTA tubes and process within 30 min of the collection. PBMCs were isolated using Histopaque (1	50% RPMI 1640 medium, 40% FBS and 10% DMSO and frozen slowly to -80°C in Mr Frosty box for 4	37°C (10 min) and then centrifuged (5000 × g), washed and centrifuged again before suspension in PBS	Increased level of DNA strand breaks (4, 6, 8, 10 and 12 months) and Fpg-sensitive sites (8, 10 and 12 month) in stored as compared to fresh samples (time zero)	[32]

subject)	h. Stored for 2, 4, 6, 8, 10 or 12 months			
Heparin tubes and isolation of PBMCs by Histopaque 1077 (20 subjects). *Note*: the authors call it "leukocytes" but Histopaque 1077 is used for PBMCs	Stored for 24 h in 90% FBS and 10% DMSO at -80°C (cooling rate: -1°C/min)	Thawed in water (37°C) for 20 min and then centrifuged for 3 min (1500 rpm) and resuspended in FBS	Higher levels of DNA strand breaks in frozen than fresh samples (tail moment values 3.3 versus 0.4, respectively). The extent of DNA damage cannot be assessed, although maximal levels of DNA migration is 198 tail moment values. Assuming a linear relationship between tail moment and %tail DNA (and that the maximal tail moment value is similar to 100% tail DNA), the net difference between fresh and cryopreserved samples (2.9 tail moment) corresponds to a net difference that is less than 2% tail DNA	[25]
Heparin tubes and blood mixed with RPMI 1640 medium and PBMC collection by Histopaque 1077 from 18 subjects.	Stored in 40% RPMI, 50% FBS and 10% DMSO at -80°C for 12 months (frozen slowly for 3 h in Mr Frosty box)	Thawed and centrifuged (washed twice with RPMI 1640 medium and once with PBS) and resuspended in PBS	Higher level of DNA strand breaks (11.7% versus 6.3% tail DNA) and Fpg-sensitive sites (21.2% versus 11.5% tail DNA) after cryopreservation than fresh samples	[27]
Peripheral blood mononuclear cells (irradiated with ionizing radiation)	10% DMSO in Hanks Balanced Salt Solution. Stored at -80°C	Not specified	No effect on levels of DNA strand breaks in samples that were used as quality control over a 30 month period (results shown in Figure 9)	[49]
Whole blood collected in a collection bag containing citrate phosphate-dextrose. Isolation of PBMC by Ficoll gradient (blood mixed with RPMI 1640 medium)	Stored in 70% RPMI 1640 medium, 20% FBS and 10% DMSO to -80°C in Mr Frosty Nalgene boxes for 24 h and then transferred to liquid nitrogen.	Thawed in water bath 37°C for 2 min, resuspended in cold PBS with 10% FBS. Centrifuged and mixed with agarose	Unaltered levels of DNA strand breaks (levels of DNA migration approximately 3% tail DNA). Results are shown in Figure 10	NA[a]

[a]Not applicable (NA). Unpublished results by Hervé Perdry and Elisa Boutet-Robinet (personal communication).

Table 4. Effect of cryopreservation and thawing on levels of DNA damage in cells from whole blood

Collection	Freezing and storage	Thawing	Effect	Reference
Heparin tubes (5 subjects)	Stored at -20°C up to 8 days	Not specified	Storage at -20°C generated high levels of DNA strand breaks, whereas storage at 4°C or room temperature did not affect the level of DNA strand breaks	[53]
Heparin tubes. Samples from 10 subjects	Mixed 1:1 with 80% RPMI 1640 medium with 20% DMSO and cryopreserved at -80°C. Store for 4 months	37°C water bath (time not specified), centrifugated and resuspended in PBS	No difference in DNA strand breaks between fresh (4.1% tail DNA) and frozen samples (4.1% tail DNA)	[54]
Heparin tubes (1 donor)	Mixed 1:10 with 75% RPMI 1640 medium, 10% FBS and 15% DMSO and frozen slowly to -80°C. Stored for 1 month	37°C water bath	No difference in levels of DNA strand breaks between fresh (≈1200 tail moment units) and frozen samples (≈1700 tail moment units, values estimated from graph). Levels of DNA strand breaks in samples after treatment with ionizing radiation (2 Gy) showed the opposite trend (≈4000 and ≈3200 tail moment units, respectively). *Note*: the tail moment do not reveal information about the shape of the comets	[38]
EDTA tubes and immediately processed. Blood was collected from 28 subjects	Stored with or without 10% DMSO for 3 days, 1 week or 1 month at -20°C or -80°C	30 min at 4°C	Accumulation of DNA strand breaks in samples without 10% DMSO and stored at -80°C (approximately 2.0 to 6.3% tail DNA). No effect in samples with 10% DMSO. *Note*: the dataset does not contain a representative sample of freshly isolated whole blood as all samples were at least kept overnight (approximately 15 h) at 4°C	[55]
EDTA tubes from 10 subjects. Time from blood	At -20°C or -80°C Storage time not	Thawing not specified, but the cells were	Slightly higher level of DNA strand breaks in cryopreserved whole	[26]

collection to storage is not specified	reported	subsequently mixed with PBS and centrifuged. Cell pellet resuspended in PBS and used in the comet assay	blood than fresh samples (1.8% versus 0.5% tail DNA). Isolated leukocytes from frozen whole blood had higher levels of DNA strand breaks (10-20% tail DNA)	
Blood collected from 3 subjects in EDTA tubes and kept maximally 1 h at room temperature before processing	Small volume (200-250 µL) in cryostraws at -80°C Stored for 1, 4 or 12 weeks	Thawed and mixed directly with agarose	No differences in levels of DNA strand break and Fpg-sensitive sites (strand breaks: 3.1, 1.9, 4.3 and 3.2, Fpg-sensitive sites: 9.9, 11.7, 10.6, 12.1 %tail DNA, estimated from graph) in fresh samples and cryopreservation for 1, 4 or 12 weeks	[30]
Whole blood collected during exsanguination of rats	Blood mixed 1:20 with solution containing EDTA and 10% DMSO. Flash frozen in liquid nitrogen before being stored at -80°C for 1 or 8 weeks	Processed directly after being thawed	Higher level of DNA strand breaks in 8-weeks frozen samples (3.5% tail DNA) than 1-week (2.9% tail DNA) and fresh samples (2.7% tail DNA). Similar effect in blood cells from rats that were exposed to ethyl methanesulfonate (e.g. highest dose increased the level of DNA strand breaks in 8-wk (26.5% tail DNA) and 1-wk (30.3% tail DNA) samples as compared to fresh samples (18.0% tail DNA)	[57]
Blood collected in EDTA tubes from 4 donors and placed on ice for maximally 1 h. PBMCs isolate by Histopaque 1077	Blood mixed 1:50 with RPMI 1640 medium with 10% FBS, 10% DMSO and 1 mM deferoxamine. Stored for 1, 7, 14 or 28 days at -20°C or -80°C (1°C/min)	Directly mixed with agarose (thawing procedure not specified)	No difference in DNA strand breaks between fresh and frozen samples that were stored at -80°C (tail moment ≈ 1). Accumulation of DNA strand breaks in samples that were stored at -20°C (tail moment >2). Same effects were observed for the determination of oxidatively damaged DNA, using the Fpg-modified comet assay. *Note*: the quality of the experiments cannot be assessed because there is no information about	[28]

			positive controls in the Fpg-modified assay and the tail moment values give little information about the extent of DNA migration	
EDTA tubes (4 rats)	Blood mixed 1:1 with RPMI 1640 medium with 10% DMSO (stored at -20°C for 24 h and subsequently at -80°C for 4 months)	37°C water bath, followed by centrifugation and washing twice in PBS	No difference in DNA strand break levels between fresh and frozen samples (34.5% and 33.7% tail DNA, respectively). *Note:* The level of DNA strand breaks appears to be rather high. It is stated that % tail DNA has been measured with Perceptive Instruments version IV image analysis. However, visual scoring using the five-class system indicates a very low level of DNA damage (1.2 and 1.2 arbitrary units, respectively; 0-400 arbitrary unit range). The results have been considered to be valid for the purpose of this paper, although concerns pertain to the to the presumed difference between image analysis and visual scoring	[37]
Heparin tubes from 30 subjects. Samples were processed within 6 h after isolation of blood (kept at room temperature during bench time)	Blood shortly centrifuged to remove plasma and then mixed with RPMI 1640 medium, 20% FBS and 10% DMSO (proportion 1:1 between isolated blood and freezing medium). Frozen to -80°C in Mr Frosty Nalgene boxes and stored 12 months.	37°C water bath	No difference in DNA strand breaks between fresh and frozen whole blood (4.0 versus 4.2% tail DNA). "Fpg buffer control" (i.e. samples incubated in Fpg buffer) had slightly higher level of DNA strand breaks in frozen whole blood than fresh samples (6.4% versus 4.9% tail DNA). It might indicate that certain cryopreservation-generated DNA lesions are converted to strand breaks during the 1 h enzyme treatment period Lower levels of Fpg-sensitive sites in frozen (14% tail DNA) than	[29]

			fresh whole blood samples (18.2% tail DNA)	
Blood collected in EDTA tubes and immediately frozen (30 subjects)	Frozen at -80°C. Stored for 12 months	Thawed (maximally 5 min) at 37°C and mixed directly with agarose	No difference frozen (10.4% tail DNA) and fresh (9.2% tail DNA)	[58]
EDTA tubes and process within 30 min of the collection (1 subject)	Frozen at -80°C (250 µL). Stored for 2, 4, 6, 8, 10 or 12 months	Thawed on ice at room temperature (20-23°C)	Increased level of DNA strand breaks (4, 6, 8, 10 and 12 months) and Fpg-sensitive sites (6, 8, 10 and 12 month) in stored as compared to fresh samples (at time zero)	[32]
Blood collected in EDTA tubes (60 subjects)	EDTA tubes and directly frozen at -80°C. Stored for 5 years	37°C water bath (2-3 min) and directly processed in the comet assay	Higher level of DNA strand breaks in frozen samples (4.4% tail DNA, n = 60, men only) as compared to fresh samples (1.6% tail DNA, n = 162, both men and women). *Note*: frozen and fresh samples were not from the same donors	[60]

Table 5. Effect of cryopreservation and thawing on levels of DNA damage in buffy coat samples

Collection	Freezing and storage	Thawing	Effect	Reference
EDTA tubes from 3 subjects and kept maximally 1 h at room temperature before processing	Small volume (200-250 µL) in cryostraws at -80°C. Stored for 1, 4 or 12 weeks	Thawed at 37°C and mixed directly with agarose	Increase levels of DNA strand breaks, Fpg-sensitive sites and H_2O_2 sensitivity after 12 weeks of cryopreservation as compared to fresh samples	[30]
EDTA tubes (9 subjects)	Snap (-80°C). Stored a few days	37°C water bath and placed on ice	Higher levels of DNA strand breaks in frozen buffy coat than fresh PBMCs (authors argue that the effect is negligible as the medians are less than 2% tail DNA). Higher levels of Fpg-sensitive sites in isolated leukocytes from frozen buffy coat (45% tail DNA) than whole buffy coat and fresh PBMCs (approximately 29% tail DNA for both samples). *Note*: DNA damage levels were not assessed in cryopreserved PBMCs. As the buffy coats are processed (washing and centrifugation), it is not possible to distinguish the effect of cryopreservation from one of the cell isolation procedures	[61]

Figure legends

Figure 1. Mean of DNA strand break in peripheral blood mononuclear cells (PBMCs) from six subjects (three with normal weight and three overweight). PBMCs were analysed as fresh or frozen samples. DNA strand breaks in fresh samples were only assessed at time 0, 1 and 2 h. The frozen samples were analysed directly after thawing and after culture at 37°C. After thawing the samples quickly, PBMCs were centrifuged at 4°C, 750 × *g* for 5 min and then the cell pellet was resuspended in PBS for washing. After washing, PBMCs were resuspended in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine and then split in a 48-well-plate (approximately 400,000 cells/well). Cells were used in comet assay either immediately or after thawing at 1, 2 and 16 h. Unpublished results, Helga Stopper, University of Wurzburg, Germany.

Figure 2. Distribution of cryopreserved PBMCs directly after thawing, after 1, 2 and 16 h incubation at 37°C in different DNA damage categories. The results in different damage classes are from 100 cells of each of the samples from the same six subjects as shown in Figure 1 (means and standard error of the mean; unpublished results by Helga Stopper and Ezgi E. Bankoglu, University of Wurzburg, Germany).

Figure 3. DNA strand breaks in peripheral blood mononuclear cells after simulated "shipping" condition. The samples were frozen in MEM with 10% DMSO, 5% antibiotics and 10% FBS and kept at -80°C for 16 h or analysed as fresh samples ("fresh"). The visual score for all four fresh samples were zero arbitrary units (a.u.). The cryopreserved samples were transferred to dry ice for 0, 24, 48 or 72 h before analysis. Lastly, a subset of samples were stored on dry ice for 48 h and subsequently transferred to -80°C for 1 week before analysis. The columns are mean and standard error of mean from four repeats from one

healthy donor. The level of DNA strand breaks is reported as visual score in arbitrary units (0-400 a.u.).

Figure 4. Levels of DNA strand breaks in mouse lymphoma cells, used as cryopreserved assay controls in a human biomonitoring study [39]. The cells were cryopreserved at -80°C in RPMI 1640 medium with 50% FBS and 10% DMSO at -80°C. The original report only summarized the mean and standard deviation for the assay controls. The regression line shows a weak decline, but it is not statistically significant (r = 0.23, P = 0.09, linear regression). In comparison, the level of DNA strand breaks in PBMCs from healthy human donors ranged from 5.9 to 10.2 tail moment units (data not displayed).

Figure 5. DNA strand break levels in pre-made slides that were produced to participating laboratories in the ECVAG ring-trial in 2007 ("original") and the same batches of cells that were analysed again in 2020 after approximately 13 years of storage at -80°C, using 50% foetal bovine serum, 40% RPMI 1640 medium and 10% DMSO as freezing medium ("old"). "New (2020)" symbols are results from new experiments on γ-irradiated THP-1, performed in 2020. The comets have been scored using Comet Assay IV software from Perceptive Instruments in five different laboratories in the original assessment and the central laboratory in 2020.

Figure 6. Levels of DNA strand breaks (solid symbols) and Fpg-sensitive sites (open symbols) in HeLa cells treated with 1 µM Ro + 4 min light before cryopreservation. The results are from two different batched of cryopreserved cells (represented by triangles and squares). There is no effect of storage on levels of DNA strand breaks (r = -0.17, P = 0.54) and Fpg-sensitive sites (r = 0.29, P = 0.22, linear mixed effect model with batch as categorical factor). Results obtained by Amaya Azqueta, University of Navarra (previously unreported results).

Figure 7. DNA damage levels in HL-60 cell control samples in a study on the effect of bariatric surgery where patients were tested before and 12-months after surgery. The samples were obtained from the freezer at 1-14 months and used as reference controls in the comet assay. Extra slides had been prepared and for the evaluation shown here; these were strained and scored on the same day. The linear regression lines are not statistically significant (DMSO: r = 0.20, P = 0.50; MMS: r = 0.22, P = 0.46). The samples were procured for the study reported by Bankoglu et al. [47], whereas the results shown here are previously unpublished. Treatment of the cells was either with 1% DMSO or with 100 µM MMS for 4 h, then samples were cryopreserved in 10% DMSO containing medium and stored at -80°C.

Figure 8. Difference in DNA strand break levels between fresh and cryopreserved (at -80°C) peripheral blood mononuclear cells (PBMCs) and whole blood. The time-dependent effects in whole blood and PBMCs have been fitted to linear curves for illustrative purpose. The results have been compiled from various publications that are cited in the main text [25;27-30;32;37]. Results on DNA damage in whole blood by Pu and co-workers have been recalculated from tail moment levels to net difference in % tail DNA, using fold-difference in the original publication and the intercept of the linear regression line.

Figure 9. Levels of DNA strand breaks in human peripheral mononuclear blood cells, used as external quality control samples in clinical trial [49]. The cells were exposed to 20 Gy of ionizing radiation and cryopreserved at -80°C in Hanks Balanced Salt Solution with 10% DMSO. Levels of DNA strand breaks in un-irradiated cells were 3.8% tail DNA.

Figure 10. Levels of DNA strand breaks in PBMCs at 8-88 weeks after cryopreservation. The data are depicted as two different strata because the Trevigen slides were used in the first period (weeks 8-40) and Gelbonds in the second period (weeks 44-88). The results are not published, but personal communication by Hervé Perdry and Elisa Boutet-Robinet.

Figure 1

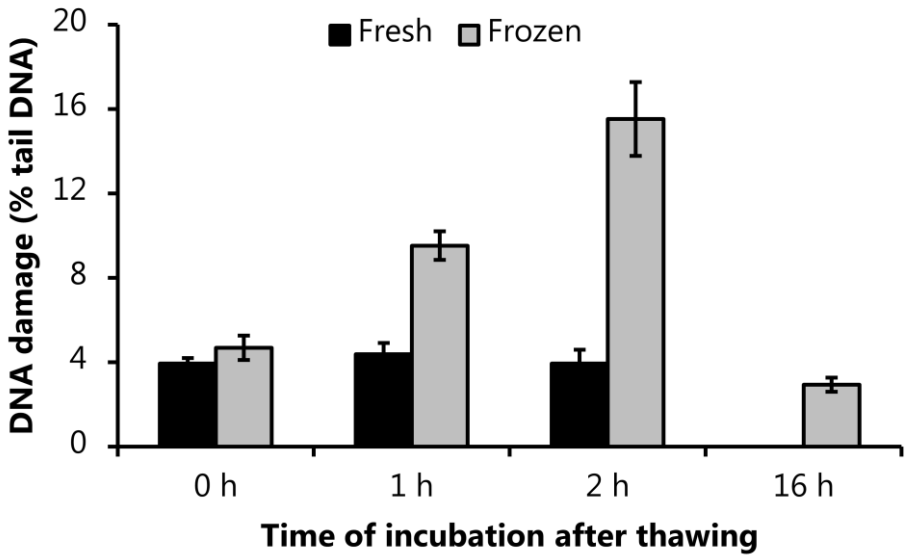
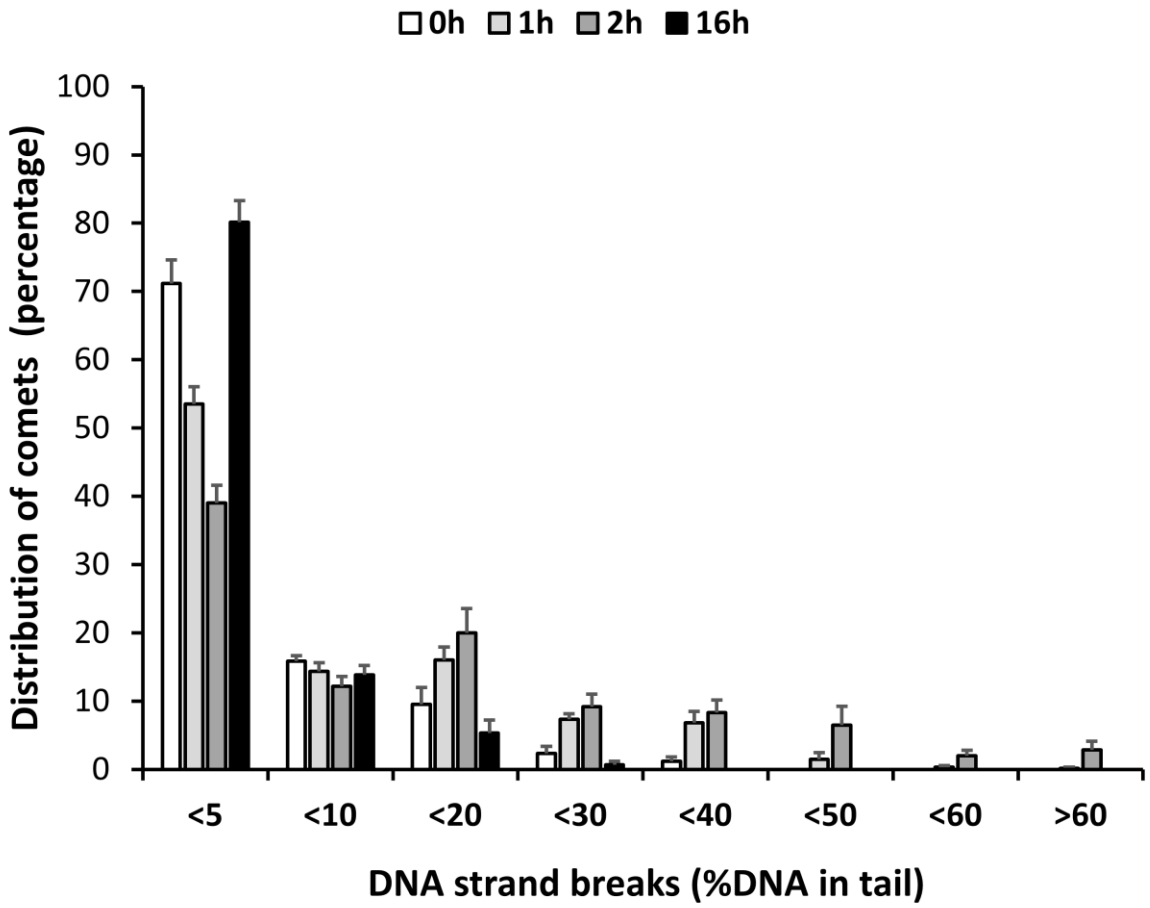


Figure 2



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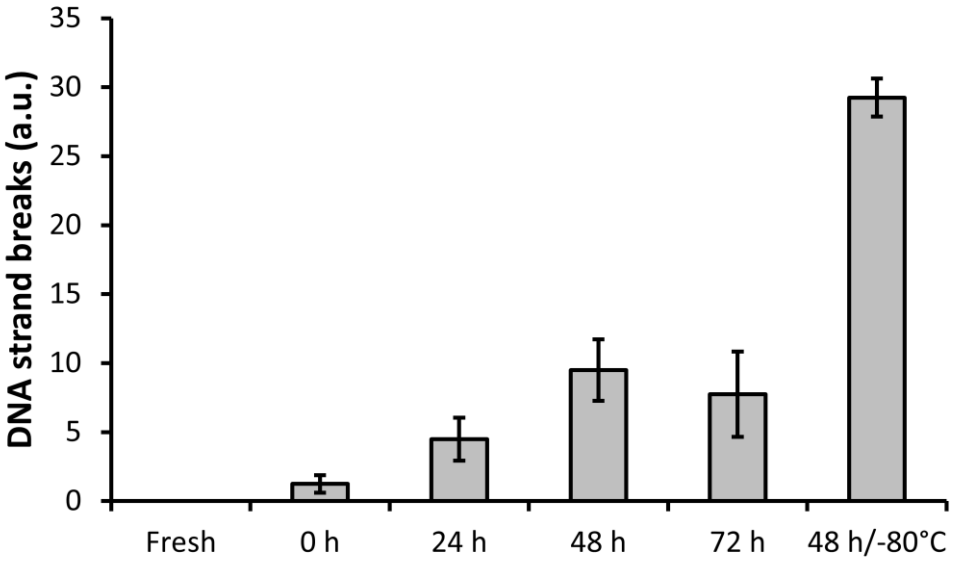

Figure 4

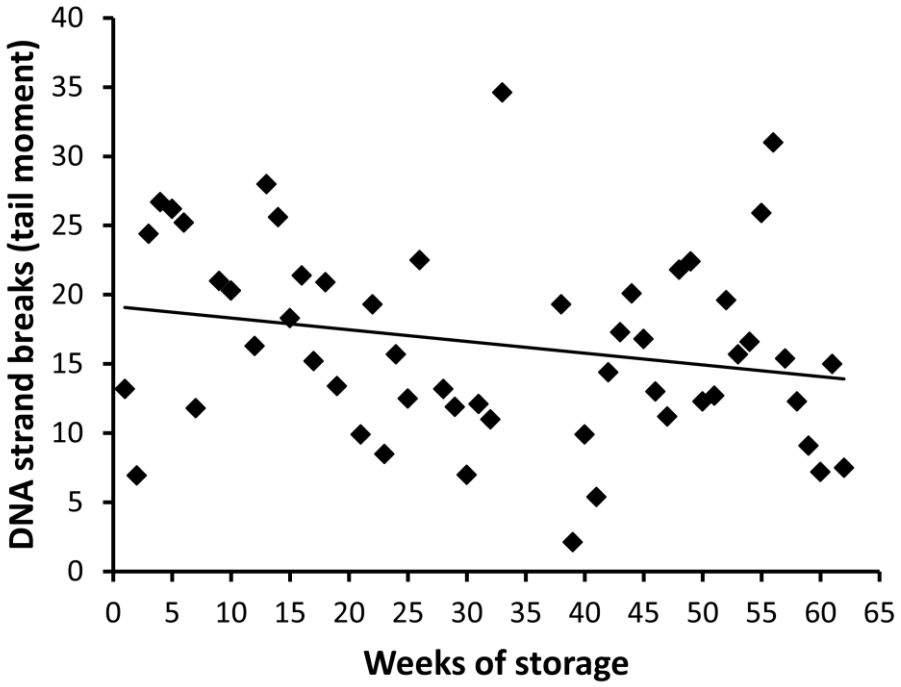

Figure 5

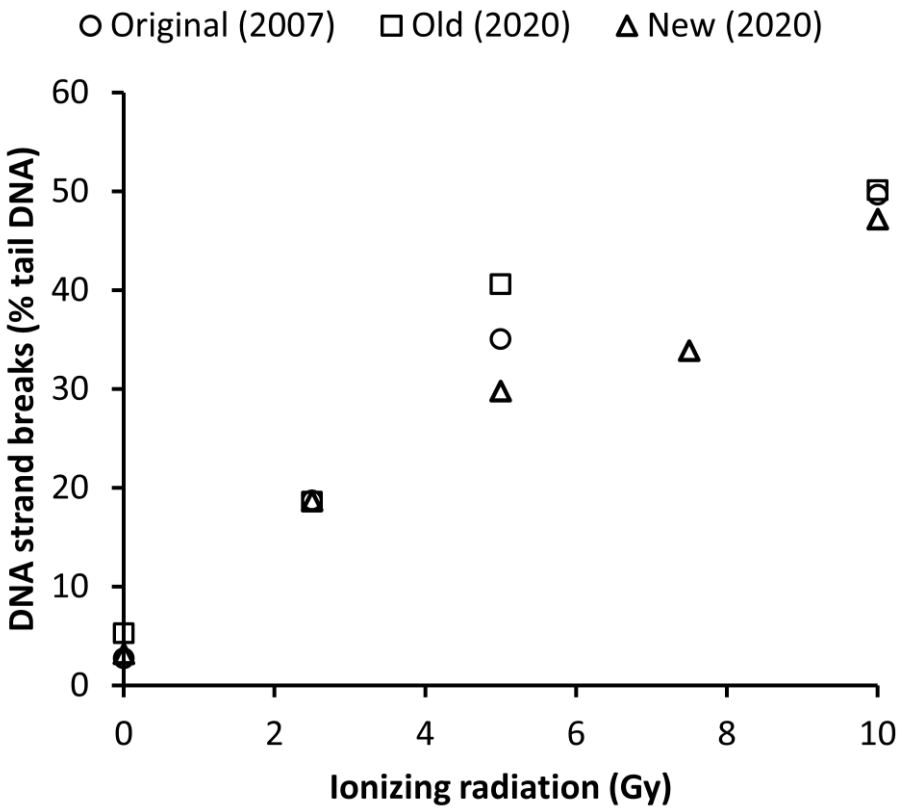

Figure 6

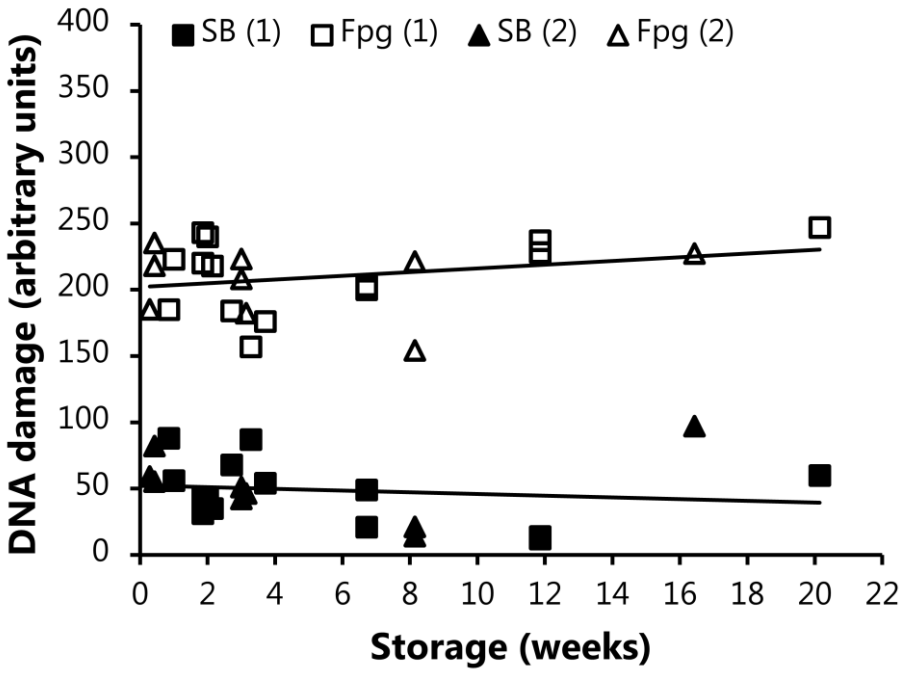

Figure 7

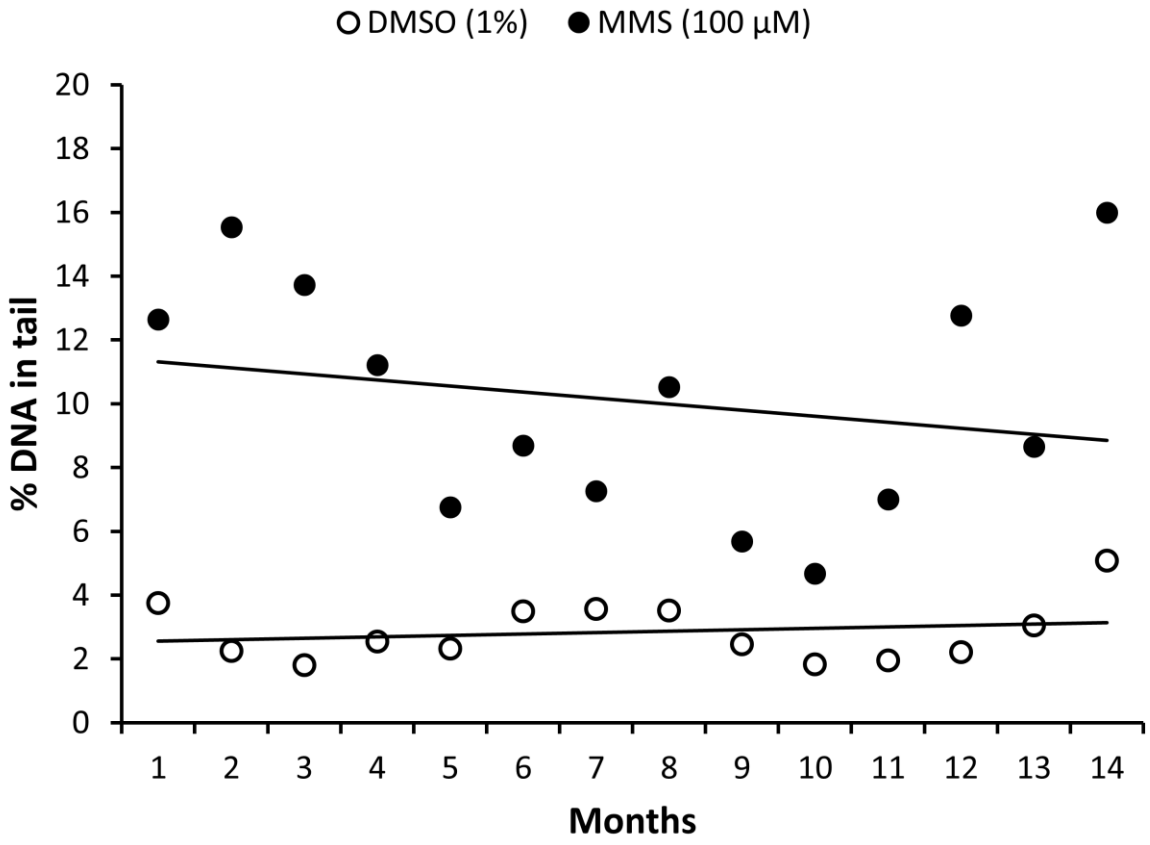

Figure 8

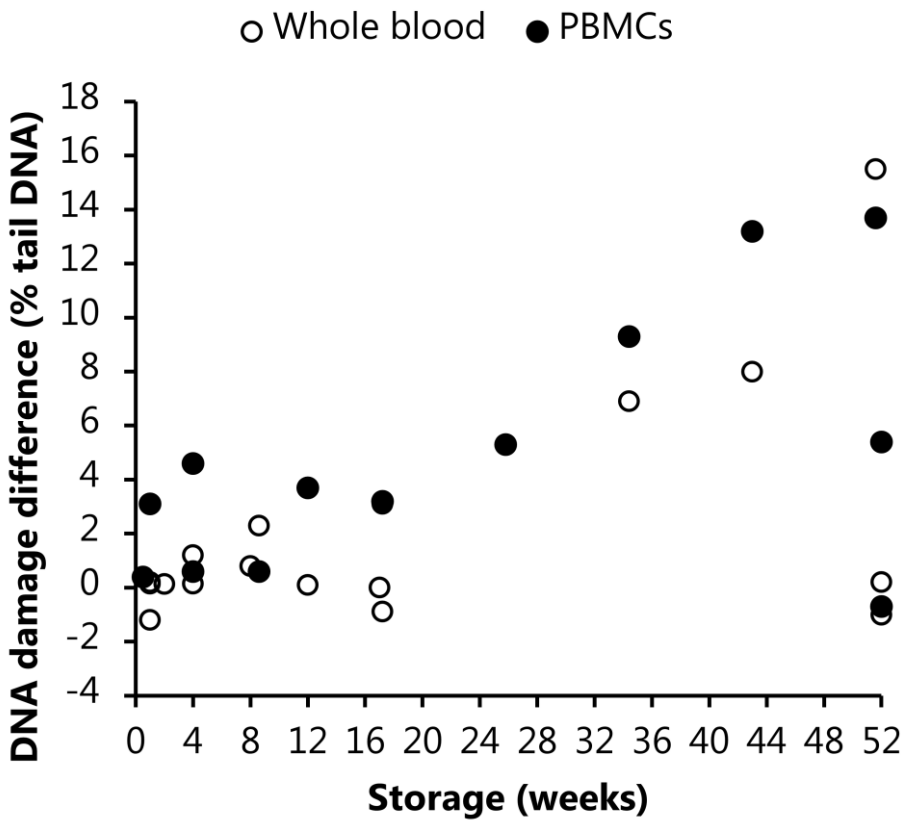

Figure 9

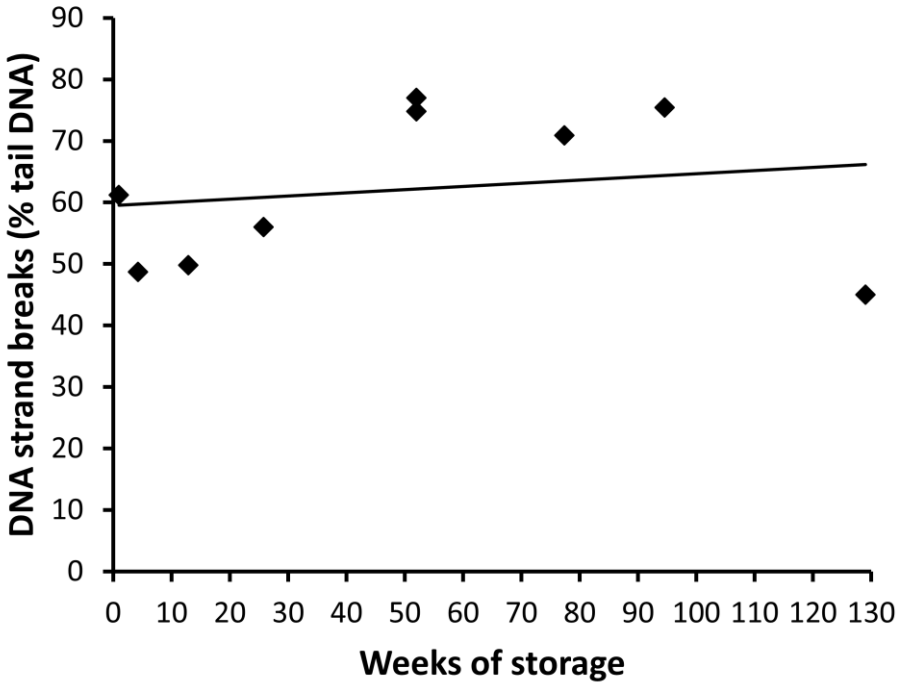

Figure 10