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Original Contribution

Simplified method for the collection, storage, and comet assay analysis of DNA damage in whole blood

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ABSTRACT

Single-cell gel electrophoresis (comet assay) is one of the most common methods used to measure oxidatively damaged DNA in peripheral blood mononuclear cells (PBMC), as a biomarker of oxidative stress in vivo. However, storage, extraction, and assay workup of blood samples are associated with a risk of artifactual formation of damage. Previous reports using this approach to study DNA damage in PBMC have, for the most part, required the isolation of PBMC before immediate analysis or freezing in cryopreservative. This is very time-consuming and a significant drain on human resources. Here, we report the successful storage of whole blood in ~250 μl volumes, at −80 °C, without cryopreservative, for up to 1 month without artifactual formation of DNA damage. Furthermore, this blood is amenable for direct use in both the alkaline and the enzyme-modified comet assay, without the need for prior isolation of PBMC. In contrast, storage of larger volumes (e.g., 5 ml) of whole blood leads to an increase in damage with longer term storage even at −80 °C, unless a cryopreservative is present. Our "small volume" approach may be suitable for archived blood samples, facilitating analysis of biobanks when prior isolation of PBMC has not been performed.

Oxidatively damaged DNA is one of the most widely measured biomarkers of oxidative stress [1]. Highlighted among the findings of the European Standards Committee on Oxidative DNA Damage was the possible formation of adventitious damage during DNA extraction and workup [2]. As a result, attention has focused on analysis methods and storage conditions that minimize such risk. Although assays such as alkaline single-cell gel electrophoresis (comet assay) have consequently proved popular [3], the prevention of artifactual formation of damage during sample storage remains largely unaddressed. Peripheral blood mononuclear cells (PBMC) are frequently used as surrogates in which to measure DNA damage. However, literature reports seem to advocate the isolation of PBMC from whole blood before freezing in a suitable cryopreservative, to prevent formation of adventitious damage. This involves the labor-intensive and time-consuming step of PBMC isolation, invariably using density gradient centrifugation, largely ruling out the use of archived samples stored in the majority of biobanks.

As a consequence, it seems that most of the reports in the literature have used isolated PBMC in conjunction with comet assay (for example, McNamee et al. [4], Sirota and Kuznetsova [5], Braz and Salvadori [6]). However, a number of reports have emerged that suggest whole blood could be used directly in the comet assay, obviating the need for prior isolation of cells [7–12]. Although this represents an improvement, many of these reports have required the introduction of steps in addition to the process of phlebotomy, such as dilution of blood samples with RPMI [7] or formation of a "sandwich" of varying concentrations of agarose on the comet slide [10,11], before the comet assay. These reports have also involved venipuncture and collection of significant volumes of blood, despite requiring only a few tens of microliters in the comet assay. Storage of whole blood and PBMC entails the use of cryopreservatives, such as dimethyl sulfoxide (DMSO) [13], introducing another step into the analysis procedure. The procedure would be simpler if (i) additional steps were not required, (ii) smaller volumes of blood were collected, and (iii) blood samples could be frozen without cryopreservative.

There is a clear need for simple, reliable assays of DNA damage, for example, in human biomonitoring studies, and the comet assay offers one solution to this. Although significant advances are being made in the reduction of variability in the comet assay [14,15], there remains a paucity of data on the use of small volumes of whole blood for damage assessment and the effect of storage conditions on artifactual formation of damage. Investigating these issues could make the

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comet assay more amenable to "field studies" of the kind associated with human biomonitoring. We therefore investigated the applicability of alkaline and enzyme-modified comet assay to a whole-blood source (venous vs capillary), varying the volume of blood collected, plus evaluating the effects of various common storage temperatures.

Materials and methods

Human 8-oxoguanine DNA glycosylase 1 (hOGG1) was purchased from New England Biolabs (Hitchin, UK). Low-melting-point (LMP) agarose was obtained from Invitrogen (Paisley, UK) and normal-melting-point (NMP) agarose from Helena Biosciences Europe (Tyne and Wear, UK). Unless stated otherwise, all other materials and chemical reagents were from Sigma Chemical Co. (Dorset, UK). Lancets were obtained from Owen Mumford Ltd. (Oxford, UK).

Cell culture

To study the possible effect of lower cell number (equivalent to the number present in the smaller volumes of whole blood studied here) on DNA damage, human epithelial RT112 bladder cancer cells (ATCC, Manassas, VA, USA) were used. These cells were grown in Eagle's minimum essential medium supplemented with 10% (v/v) fetal calf serum and 1% nonessential amino acids (Invitrogen) at 37 °C in an incubator with a humidified atmosphere, comprising 95% air/5% CO_2. Decreasing numbers of cells (final numbers 15,000, routinely used in comet assay, to 300) were placed on NMP agarose-precoated microscope slides in 80 μl LMP agarose. The remainder of the protocol was as for the standard comet procedure (described below).

Blood collection and storage conditions

Unless otherwise stated, human blood samples were collected by venipuncture using a 21-gauge needle and syringe, from the antecubital vein (~5 ml), and immediately transferred into tubes containing sterile EDTA solution (to a final concentration of 1.6 mg EDTA/ml of blood). Blood samples collected by lancet (~250 μl) were obtained from the side of a finger and transferred, by pipette, into tubes containing sterile EDTA solution (0.4 mg EDTA/250 μl of blood). Blood was processed immediately for comet assay, kept in a fridge, or placed in a freezer and left to freeze, without the use of any "freezing device." Storage temperature was 4, −20, or −80 °C, with or without 10% DMSO, for the following time periods: overnight (i.e., 15 h), 72 h, 1 week, or 1 month. Samples stored at −20 and −80 °C, irrespective of whether they were in DMSO, were allowed to thaw for up to 30 min at 4 °C, as required, before proceeding with the comet assay as for unfrozen cells.

Treatment of PBMC with H_2O_2

To evaluate the effect of storage on induced levels of DNA oxidation in PBMC, whole blood was treated with H_2O_2 and then stored at −80 °C for up to 1 week, as described above. The H_2O_2 was diluted in ultrapure water to prepare a 10 mM stock solution, which was added to 100 μl whole blood to final concentrations of 50, 100, 200, 500, and 1000 μM and incubated for 30 min on ice in the dark. For untreated cells, the incubation on ice was carried out with PBS in place of H_2O_2.

Enzyme-modified and alkaline comet assay for whole blood

The alkaline comet assay used here was adapted from the method described previously [16] with slight modifications as follow:

To optimize cell concentration in the comet assay, we investigated various combinations of blood volume (5, 10, 20, and 100 μl) and two concentrations of LMP (0.6 and 1.2% w/v). The above volumes of blood were suspended in 200 μl of prewarmed LMP agarose, from which 80 μl was dispensed directly to each half of a microscope slide (precoated with 1% NMP), and the two gels per slide were allowed to set under a coverslip, on ice. After an overnight lysis step, the slides were washed once with cold double-distilled water followed by rinsing in two changes of enzyme reaction buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg/ml BSA, pH 8 adjusted with KOH), each for 10 min at room temperature. The enzyme reaction buffer was prepared as a 10× stock and frozen at −20 °C in aliquots. To detect purine-derived lesions, 50 μl of hOGG1 in enzyme reaction buffer was used, resulting in a final concentration of 0.16 U per gel [16]. On the other gel of the slide, 50 μl of enzyme reaction buffer alone was applied. Slides were incubated for 45 min at 37 °C in a humidified incubator. All subsequent steps were according to the previously described method [16]. Fifty comets per gel were selected at random and scored using a fluorescence microscope (Axioskop; Carl Zeiss, Jena Germany) fitted with an excitation filter of 515–535 nm and a 590 nm barrier filter at 20× magnification. Giovannelli et al. [8] noted that polymorphonuclear leukocytes give nonhomogeneous comet tails. However, these may be easily identified by microscopy [8] and were avoided during scoring, in preference for mononuclear leukocytes. Percentage tail DNA of the comets was recorded, using Comet Assay IV analysis software, version 4.2 (Perceptive Instruments, Haverhill, Suffolk, UK).

Ethics

Ethics approval for blood collection from healthy volunteers used in this study was granted by the University of Leicester's College of Medicine, Biological Sciences and Psychology Committee for Research Ethics Concerning Human Subjects (Non-NHS).

Statistics

Statistical analysis of data was performed using Prism version 5 software (GraphPad Software, San Diego, CA, USA), one-way ANOVA, and post hoc Bonferroni's multiple comparison tests in which $P<0.05$ was regarded as significant. Each experiment was performed on a minimum of two separate occasions, with the exception of the H_2O_2 study.

Results

Optimized comet assay for whole blood

The following experiments were performed on human blood obtained by venipuncture unless otherwise stated.

It was necessary to optimize the volume of whole blood and LMP gel concentration for use in the comet assay, to achieve enough cells for scoring and still allow the agarose to set. Using blood obtained and immediately processed for comet assay, we noted distorted morphology with increasing volumes of blood, particularly at higher (100 μl) volumes of blood (Figs. 1A and B). Even the use of 20 μl of whole blood produced nucleoids with a severely disrupted morphology (data not shown). The lowest levels of damaged/disrupted nucleoid morphology were seen with the smallest volume of blood (5 μl), while still providing enough nucleoids for scoring (Figs. 1C and D).

A similar pattern was noted using the above conditions and examining the effect of overnight storage by alkaline comet assay (Fig. 2). We noted a consistent trend toward greater apparent adventitious damage with increasing volumes of blood, and again 5 μl of blood was deemed to be optimal. As expected, embedding cells in 1.2% LMP appeared to impede migration of tail DNA (irrespective of cell number used), compared to electrophoresis in 0.6% LMP. For the largest volume of blood used (20 μl), overnight storage at 4 °C tended to lead to an increase in damage, compared to storage at −20 or −80 °C. However, this effect was less discernable when using 5 and

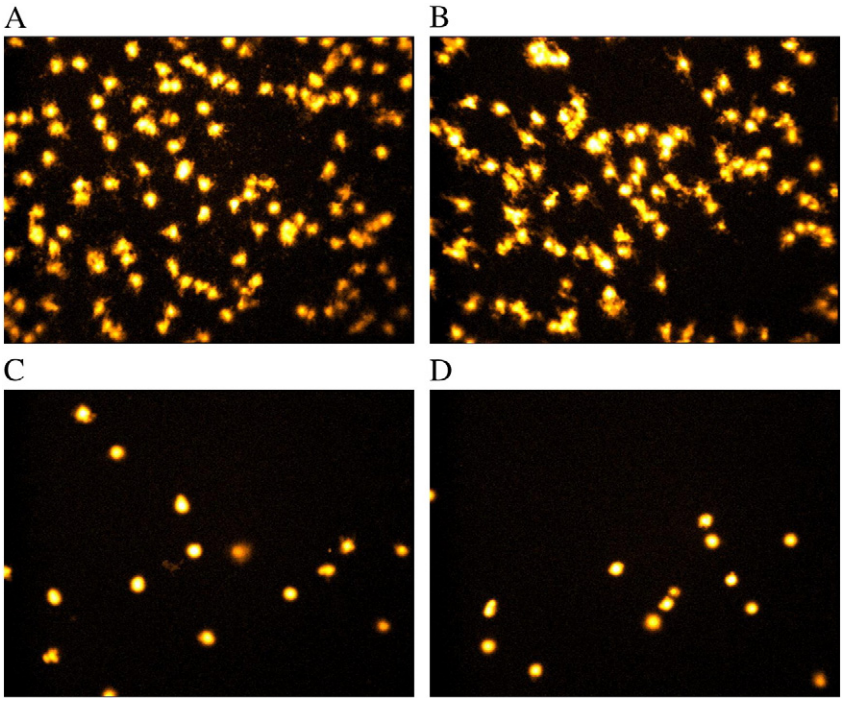

Fig. 1. Effect of LMP gel concentration on peripheral blood mononuclear cell morphology, adding 100 µl of whole blood to 200 µl of (A) 0.6% or (B) 1.2% LMP agarose and 5 µl of whole blood to 200 µl of (C) 0.6% or (D) 1.2% LMP agarose and treating for comet assay.

10 µl of blood. On this basis, the optimal conditions were determined to be 5 µl of whole blood embedded in 0.6% LMP agarose.

We also performed a comparison between our technique for comet assay using whole blood with a method using "micro" Ficoll density gradient isolation to prepurify PBMC before comet assay as reported by Singh [17]. However, this approach was shown to report higher levels of background damage, compared to our method (~8–10% tail DNA; data not shown), and was not pursued further.

Using small volumes of blood (and hence a small number of cells) may increase the likelihood of scoring cells toward the edge of the gel, with the attendant risk of artifactual increases in damage [18]. RT112 cells in various cell numbers per gel (300, 500, 1000, 3000, 5000, 10,000, and 15,000) were used to evaluate whether the lower number of cells in 5 µl of blood may be associated with such increased risk of artifact during scoring. When scoring was performed randomly across the entire gel, mean levels of damage increased significantly with decreasing cell number (Fig. 3, unrestricted). In contrast, when scoring was restricted to within the central part of the gel, no significant increase was noted (Fig. 3, restricted). This shows that 5 µl of blood, containing ca. 5000 cells, provides a sufficient number of comets that can be scored within the central ninth of the gel, where edge effects are negligible.

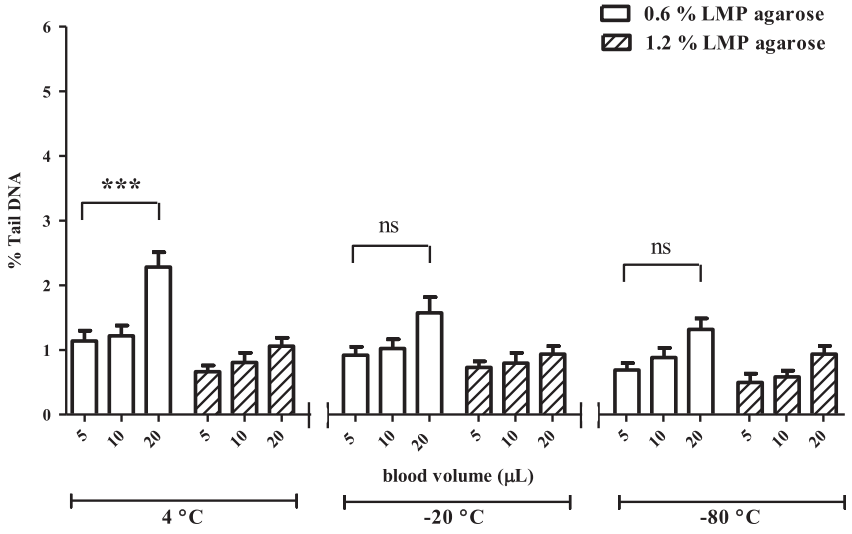

Fig. 2. Determination of optimal blood volume and LMP gel concentration. After an overnight (12 h) incubation of whole blood (5 ml; in anticoagulant) at 4, −20, and −80 °C, the optimization was performed on various volumes of human whole blood (5, 10, and 20 µl) in conjunction with 200 µl of 0.6 or 1.2% LMP agarose gel. DNA damage was expressed as % tail DNA after analysis by alkaline comet assay. The results represent the means ± SEM of two individual experiments ($n = 200$ determinations) with blood samples from a single donor (***$P < 0.001$; ns, not significant).

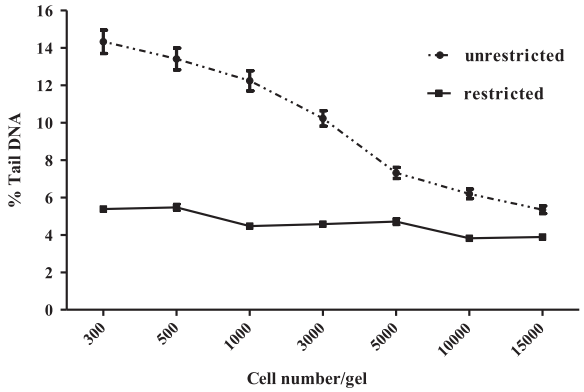

Fig. 3. Effect of cell number per gel on DNA damage scoring in a RT112 cell line. Comet assay was performed on gels containing 300–15,000 cells/gel and scored by including cells from across the entire gel (unrestricted) or with strict limits of the central one-ninth of the gel (restricted). DNA damage was expressed as % tail DNA. The data represent the means ± SEM of three individual experiments.

Effect of storage time/temperature on endogenous levels of DNA damage

Using the above optimized assay conditions (i.e., 5 μl whole blood, 0.6% LMP, scoring restricted to central part of the gel), the effects of storage time and temperature on endogenous levels of damage were investigated. We noted a significant increase ($P<0.001$) in damage after storage of whole blood in 5 ml volumes for 1 month, irrespective of storage temperature, although the lower the temperature (both −20 and −80 °C) the smaller the increase in adventitious damage (Fig. 4A). We noted a similar pattern of damage formation after storage of samples in DMSO, at 4 and −20 °C (Fig. 4B). However, there was no apparent increase in damage after storage for 1 month at −80 °C in the presence of DMSO (Fig. 4B).

Effect of storage on induced levels of DNA damage

Blood samples treated with H_2O_2 immediately after collection were used as a positive control demonstrating the dose-dependent induction of hOGG1-sensitive sites, but not alkali-labile sites/frank strand breaks, using the hOGG1-modified comet assay (Fig. 5A). We were able to produce very similar dose–response curves, using the

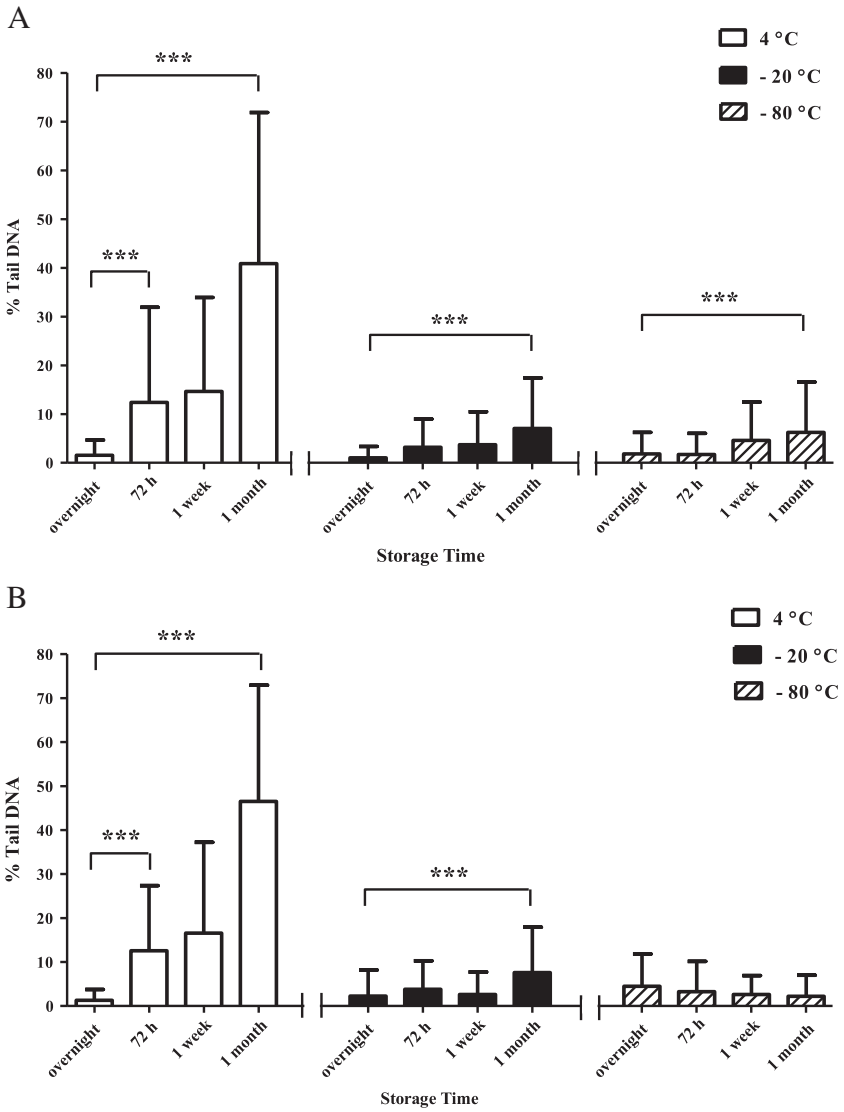

Fig. 4. DNA damage in whole blood (5 ml) after various storage times and temperatures, (A) without and (B) with DMSO. Comet assays were performed using optimized conditions. Storage conditions were overnight (12 h), 72 h, 1 week, and 1 month at 4, −20, and −80 °C. Data are presented as means ± SD of more than eight individual experiments (***$P<0.001$).

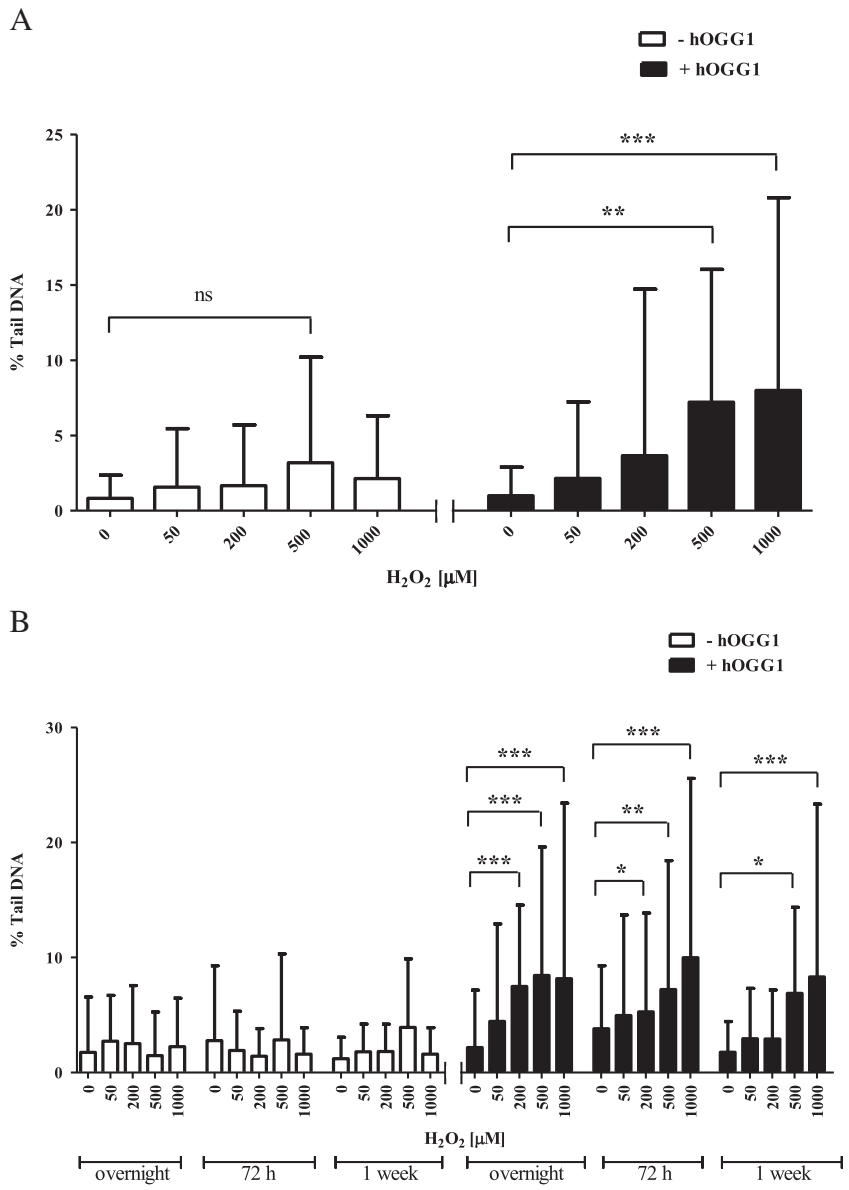

Fig. 5. Dose–response for whole blood treated with H_2O_2 (0–1000 μM) and analyzed (A) immediately or (B) after storage at −80 °C overnight or for 72 h or 1 week, without DMSO. DNA damage was expressed as % tail DNA after analysis by modified alkaline comet assay without or with hOGG1. The results represent means ± SD of a single experiment (*P<0.05, **P<0.01, and ***P<0.001).

hOGG1-modified comet assay, after storage of H_2O_2-treated whole blood overnight and up to 1 week at −80 °C, without DMSO (Fig. 5B). There appeared to be little difference in the levels of damage between the various storage times.

Applicability of "pin-prick" blood sampling and storage to detection of DNA damage by comet assay

To confirm the above results of storage and the use of the hOGG1-modified comet assay, and to explore the utility of smaller blood collection volumes, elements of the previous studies were repeated, but using pin-prick samples. Around 250 μl of blood, from 14 (for 4 °C storage) or 28 (for immediate assay or −20 and −80 °C storage) healthy individuals, was obtained using lancets. With no further processing the 250 μl samples were either analyzed immediately, using the optimized comet conditions described above, or stored for overnight (15 h), 72 h, 1 week, or 1 month at 4, −20, or −80 °C, without any cryopreservative. Mean baseline levels of damage did not differ between blood collected by venipuncture or lancet. As before, we noted significant increases in DNA damage, as measured by both alkaline and hOGG1-modified comet assay, in blood samples stored at 4 °C, for all time periods (Fig. 6). There was a trend toward an increase in DNA damage, after storage at −20 °C for up to 1 month, for both alkaline and hOGG1-modified comet assay, which reached statistical significance only after 1 month. In contrast no increases in DNA damage were detected, either by alkaline or by hOGG1-modified comet assay, when samples were stored at −80 °C for up to 1 month.

Discussion

The comet assay offers a number of advantages over other methods for the detection of DNA damage: (i) it is rapid, simple, and relatively inexpensive to perform; (ii) it allows for the collection of data at the individual cell level; (iii) it requires a relatively small number of cells (<10,000) in each sample; (iv) it shows a high sensitivity for the detection of DNA damage; and (v) almost all types

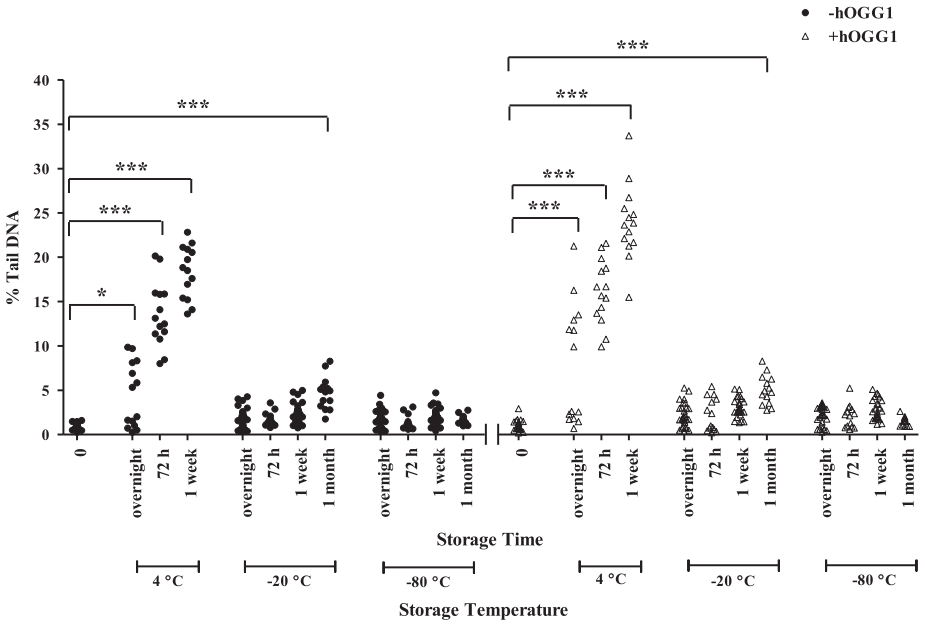

Fig. 6. DNA damage in whole blood samples collected by the lancet technique and after various storage times and temperatures. hOGG1-modified alkaline comet assay was performed on blood samples immediately and after storage overnight or for 72 h, 1 week, or 1 month without cryopreservative (at 4, −20, and −80 °C). DNA damage was expressed as % tail DNA. Each data point represents the mean of 100 determinations of damage in one blood sample from each individual ($n = 14$ for 4 °C storage or n = 28 for immediate assay or −20 °C and −80 °C storage; $^{*}P<0.05$ and $^{***}P<0.001$).

of eukaryotic cells, both in vitro and ex vivo, can be used for analysis [19]. The comet assay is most frequently used for assessing damage in cultured cells, despite having clear potential, as indicated above, to be used in human biomonitoring. The isolation of PBMC from whole blood before the comet assay has been reported [4–6,20], but this introduces a step, common to other procedures, which is time-consuming and detracts from some of the other advantages of the comet assay (speed and simplicity). Ideally, whole blood would be incorporated into the alkaline comet assay, obviating the need for prepurification, and indeed a number of groups have reported this [7,9,11,12,21], although far fewer groups have demonstrated the applicability of whole blood to the enzyme-modified comet assay [8]. The use of whole blood, and the requirement for relatively small cell numbers, has meant that skilled phlebotomy can be replaced by a simple lancet technique to obtain a suitable blood sample [21]. Mindful of these studies, and the need for simplicity in sample collection, handling, and storage, we investigated the suitability of lancet collection, various routinely used storage conditions, and the enzyme-modified comet assay analysis of whole blood.

We have established optimal agarose concentration and blood volume for the use of whole blood in the comet assay. At higher (≥1.2%) concentrations of agarose, migration of tail DNA was impeded, decreasing the sensitivity of the assay, as noted elsewhere [22]. The addition of higher volumes of blood either led to disruption of nucleoid morphology or prevented the agarose from solidifying, perhaps because of a high concentration of certain blood components or abnormally low agarose concentration. Furthermore, high cell numbers have been reported to result in overlapping comets, which can be difficult to visualize and give false-positive results [22]. Visual selection of mononuclear leukocytes rather than polymorphonuclear leukocytes is possible by the human eye during scoring; however, this may prove to be a greater challenge for an automated system.

The requirement for smaller volume blood samples (≤100–200 μl) is advantageous in that they can easily and rapidly be collected in the field using a lancet (see below) and take up significantly less storage space. We considered that smaller cell numbers, which may be associated with lower blood volumes, could lead to an apparent increase in mean levels of damage, when scoring was not strictly limited to the center of the gel. We propose that the increase in damage noted with lower cell numbers was due to the increased contribution of "edge effects"; as the number of cells per gel decreases the operator would be forced to score cells farther away from the center of the gel. We also noted higher background levels of damage in PBMC after prepurification before the comet assay, compared to analysis of whole blood. This provides further justification for our approach.

We subsequently investigated the effects of storage temperature and duration on endogenous levels of DNA damage in human whole blood samples. Unlike Anderson et al. [23], who found no difference between DNA damage in blood stored for days, at either room temperature or 4 °C, we showed unequivocally that storage at 4 °C induced significant amounts of artifactual DNA damage, irrespective of the addition of DMSO. This is similar to the findings of Narayanan et al. [24], who showed that DNA strand breakage increased significantly in lymphocytes stored at either room temperature or 4 °C, compared to freshly isolated cells. Levels of induced artifactual damage were minimized, but not entirely prevented, by storage at −20 °C both with and without DMSO. Storage of 5-ml blood samples at −80 °C, in the presence of DMSO, appeared to decrease levels of endogenous damage, which was unexpected, whereas in the absence of DMSO a small increase was noted. These findings, coupled with the report of Hininger et al. [13], suggest that larger volumes (~5 ml) of whole blood require the addition of a cryopreservative such as DMSO, to prevent an increase in damage when storing at −80 °C. Similarly, isolated lymphocytes require freezing in the presence of a cryopreservative [20], when storing for up to 2 months. This suggests that components in blood perform a function similar to that of the cryopreservative agent. The implication is that archived whole blood samples, without cryopreservative, may in fact be suitable for the comet assay analysis, albeit with greater risk of artifact with time. However, for comparative studies, in which controls have been stored in the same manner, this may not represent a significant issue. This will aid in retrospective analysis of stored samples, along with samples for which collection conditions preclude immediate analysis.

The use of the enzyme-modified comet assay is increasing, as this brings greater information on the types of lesions present in DNA via the incorporation of lesion-specific repair enzymes [18]. To evaluate the effects of storage on induced levels of oxidatively generated damage to DNA nucleobases, we pretreated whole blood with increasing concentrations of H_2O_2 before freezing for various time periods. We were able to consistently detect a dose response in nucleobase damage in the frozen samples, similar to that seen for the samples processed immediately without freezing. Although absolute levels of damage may vary between frozen and fresh samples, these data further support the potential use of archived samples for which appropriate controls exist. In contrast to H_2O_2 treatment of isolated lymphocytes or cultured cells, we did not see a dose–response increase in strand breaks. Currently we are unable to explain this finding.

Interestingly, small volumes of whole blood (~250 μl), collected by lancet, were far more amenable to longer term storage at −20 °C and in particular −80 °C, without any cryopreservative. This contrasts with the significant increase in adventitious damage when larger volumes of blood are stored, particularly without cryopreservative, as seen in Fig. 4. Similar to the results for larger volumes of blood, storage of small volumes at 4 °C, for any length of time, led to a clear increase in levels of damage, compared to samples processed immediately, and cannot be recommended. The results seen with storage of smaller volumes apply to both the alkaline and the hOGG1-modified comet assays. We speculate that this may be due to the shorter period of time required to freeze/thaw small volumes, compared to larger volumes of blood, and hence less time for damage to be generated. It is worth noting that in many biobanks whole blood is stored in small volumes, in "straws." Importantly, our data suggest that this form of storage may well be amenable to our comet method. The effects of whole blood storage in biobank straws, along with storage for periods greater than 1 month, warrant further investigation.

We noted no difference in mean basal levels of damage between fresh whole blood samples collected via venipuncture and samples collected by lancet. Furthermore, collection of these samples via a lancet is minimally invasive and exploits a major strength of the comet assay—the requirement for relatively small number of cells. This technique can be used for collection in the field and from vulnerable groups, such as neonates. Although the lancet approach can provide up to 250 μl of whole blood, only 5 μl is required for a comet assay analysis, as we have shown here.

Taken together, these findings demonstrate that small volumes of whole blood (~250 μl) collected by lancet may be stored without addition of cryopreservatives at either −20 °C for up to 1 week or −80 °C for up to 1 month without significant increases in adventitious damage. Furthermore, these samples remain suitable for direct use with our modifications of the alkaline and enzyme-modified comet assays (5 μl whole blood, 0.6% agarose, scoring of comets restricted to central part of the gel). We suggest that larger volumes of whole blood that have been archived may similarly be suitable for use in the comet assay, albeit with a caveat that background levels of damage are likely to increase with time, unless DMSO has been added. These data provide further support for the use of the comet assay in in vivo biomonitoring studies, by greatly simplifying sample collection, storage, and analysis.

Acknowledgments

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