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Comparison of DNA damage by the comet assay in fresh versus cryopreserved peripheral blood mononuclear cells obtained following dietary intervention

Cristian Del Bo', Daniela Fracassetti, Claudia Lanti, Marisa Porrini and Patrizia Riso*

Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Università degli Studi di Milano, via Celoria 2, 20133 Milan, Italy

*To whom correspondence should be addressed. Tel: +39 02 503 16726; Fax: +39 02 503 16721; Email: patrizia.riso@unimi.it

Abstract

Endogenous and oxidatively induced DNA damage, as evaluated by the comet assay, are widely used as biomarkers of oxidative stress in numerous dietary intervention studies. This analysis can be performed on fresh peripheral blood mononuclear cells (PBMCs) or on cryopreserved cells. However, information pertaining to the effects of cryopreservation on DNA damage is often missing, and this may be crucial in studies in which samples are analysed before and after intervention. The purpose of this study was to compare DNA damage in fresh versus cryopreserved PBMCs obtained from subjects following a 6-week intervention with wild blueberry drink or placebo drink. Fresh and 12-month-stored PBMCs were analysed for formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites and H₂O₂-induced DNA damage. The levels of FPG-sensitive sites were significantly higher in the cryopreserved compared with the fresh cells ($P < 0.001$), while H₂O₂-induced DNA damage was significantly lower after storage ($P < 0.001$). Both the fresh and cryopreserved samples showed reductions in FPG-sensitive sites following the wild blueberry treatment (fresh PBMCs: from $12.50 \pm 5.61\%$ to $9.62 \pm 3.52\%$, $P = 0.039$; cryopreserved PBMCs: from $22.7 \pm 6.1\%$ to $19.1 \pm 7.0\%$, $P = 0.012$). In contrast, the decrease in H₂O₂-induced DNA damage observed in the cryopreserved cells masked the protective effect of the wild blueberry drink documented in the fresh samples (fresh PBMCs: from $44.73 \pm 7.46\%$ to $36.34 \pm 9.27\%$, $P < 0.001$; cryopreserved PBMCs: from $25.8 \pm 4.6\%$ to $23.9 \pm 4.6\%$, $P = 0.414$). In conclusion, our results suggest that FPG-sensitive sites, and more importantly, H₂O₂-induced DNA damage could be significantly modified following the long-term storage of samples obtained from individuals participating in a dietary intervention study. Because storage may affect the assessment of the protective role of diet against DNA damage as a marker of oxidative stress, further research is needed.

Introduction

The comet assay is a rapid and simple technique for the evaluation of DNA damage in all types of eukaryotic cells and tissues. This assay has been used in various human biomonitoring studies to investigate the effects of exposure to pollutants, environmental contaminants (1–3) and bioactive constituents of diet and/or food (4–10). For the comet assay, cells are immobilised in a bed of low-melting-point

(LMP) agarose. Following gentle cell lysis, samples are treated with alkali to unwind and denature the DNA and hydrolyse sites of damage, and electrophoresis is then performed. Cells are successively stained with a fluorescent DNA intercalating dye and visualised under a microscope by epifluorescence (11). This assay allows for the detection of several classes of DNA alterations, including double-strand breaks, single-strand breaks, alkali-labile sites, the incomplete repair of a basic sites and cross-links (12–14).

Generally, two approaches to this assay are commonly used. The first consists of the measurement of oxidised bases generated by the use of specific enzymes, such as endonuclease III and formamidopyrimidine-DNA glycosylase (FPG) (15). Endonuclease III recognises oxidised pyrimidines, while FPG measures oxidative damage to DNA in terms of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) as well as formamidopyrimidines. Peripheral blood mononuclear cells (PBMCs) are most commonly used for biomonitoring and intervention studies. The second comet assay approach consists of the evaluation of *ex vivo* cell resistance to oxidative DNA damage induced by incubating cells with hydrogen peroxide or other stressors (16). Cell resistance is considered to be a possible marker of antioxidant status/defence (17). PBMCs are relatively easy to collect and may be considered as valid surrogates with which to measure DNA damage because they are assumed to reflect actual *in vivo* conditions (18).

Cryopreservation is commonly used for the long-term storage of different biological samples, such as tissues, embryos, gametes, sperm, cell cultures and PBMCs (19). Limited information is available with regard to the effects of freezing and storage on DNA damage in human cells, and available data are insufficient to exclude the existence of a significant impact. Several studies have revealed an increase in DNA damage upon storage compared with that found in fresh cells (20); in contrast, other studies have not observed any effects of cryopreservation on DNA damage (21). Because of these conflicting reports, it is necessary to obtain further clarification considering that, for logistic reasons, samples such as PBMCs often cannot be used immediately and require storage. The second comet assay approach is typically used in medium-long-term intervention studies.

In the present study, we aimed to compare DNA damage in fresh versus cryopreserved PBMCs obtained from subjects following a 6-week intervention with a wild blueberry drink or placebo drink. Endogenous and oxidatively induced DNA damage were evaluated using the comet assay following the intervention and after 12-month of sample storage.

Materials and methods

Chemicals

Normal-melting-point (NMP) agarose, LMP agarose, EDTA, Na₂EDTA, Tris-acetate-EDTA buffer, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), RPMI-1640 medium, Histopaque

1077, fetal bovine serum (FBS), hydrogen peroxide (H₂O₂), ethidium bromide, N-lauroylsarcosine, Triton X-100, Trizma, sodium hydroxide, bovine serum albumin and sodium chloride were obtained from Sigma (St Louis, MO, USA). Formamidopyrimidine-DNA glycosylase was kindly provided by Prof. Andrew Collins from the University of Oslo.

Isolation of PBMCs

A venous blood sample (1 × 10 ml) was collected by venepuncture from individuals with risk factors for cardiovascular disease. A total of 100 µl of whole blood was gently mixed in a microtube with 900 µl of cold RPMI-1640 medium. Then, 100 µl Histopaque 1077 was carefully added to the bottom of the tube and centrifuged at 200 × g for 4 min at room temperature. Cells were collected, and 1 ml of PBS solution was used to wash the cells. The samples were then centrifuged for 10 s at 5000 × g at room temperature to pellet the cells. The supernatants were poured off and the pellets were resuspended in PBS and immediately used or resuspended in freezing mix composed of 50% FBS and 40% RPMI-1640 supplemented with 10% DMSO as a cryoprotectant and stored at -80°C until analysis (12 months).

Experimental design

Samples were derived from a previously published dietary intervention study (22), in which 20 male volunteers received a wild blueberry drink or placebo drink for a period of 6 weeks (Figure 1). PBMCs were obtained from blood collected at the beginning and at the end of each experimental period (before/after the wild blueberry drink and before/after the placebo drink). Analysis of DNA damage (FPG-sensitive sites and H₂O₂-induced DNA damage) was performed using fresh and cryopreserved PBMCs. An aliquot of PBMCs was gently thawed in a water bath at 37°C and centrifuged to remove the media. The cells were placed on ice and washed twice with RPMI-1640 media and one with PBS. Finally, they were resuspended in 50 µl of PBS and used for subsequent analysis.

Evaluation of FPG-sensitive sites in PBMCs

The evaluation of FPG-sensitive sites was performed as previously described (6). The cell suspension was supplemented with LMP agarose (1.5% wt/vol) in Tris-acetate-EDTA buffer at pH 7.4 and 37°C and immediately pipetted (10⁴ cells per gel) onto a frosted glass microscope slide (Richardson Supply Co., London, UK) precoated

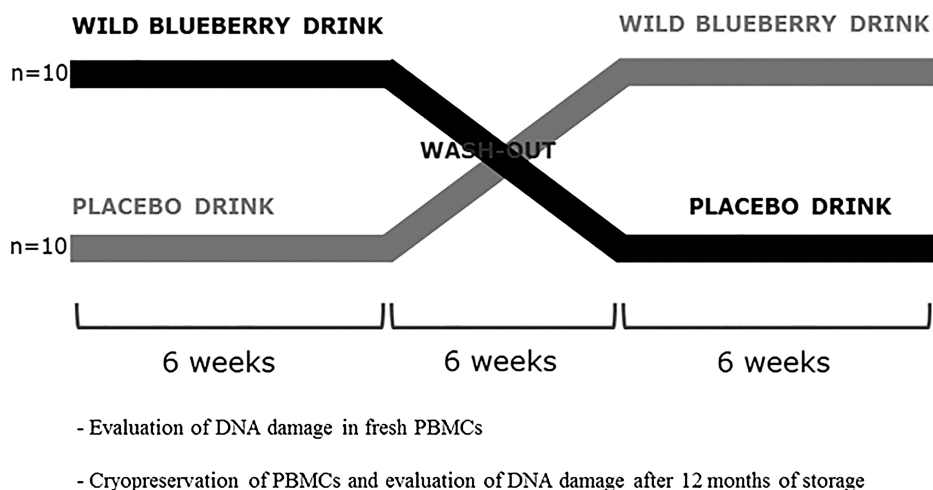


Figure 1. Randomised cross-over experimental design.

with a layer of 1% (wt/vol) NMP agarose similarly prepared in Tris-acetate-EDTA buffer. The level of oxidised bases in PBMCs was determined as FPG-sensitive sites; FPG is used to detect 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. Slides were placed in lysis buffer (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, 1% Triton X-100, 1% DMSO, and 1% *N*-lauroylsarcosine sodium salt, pH 10) for 1 h at 4°C in the dark. Two slides with two gels each were prepared for all samples. After lysis, the gels were washed in buffer (40 nM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin, pH 8). One slide was treated with FPG (100 ng/ml in buffer), and the other slide (in buffer without FPG) was used as a control. Incubation was performed at 37°C for 45 min. The slides were then transferred to electrophoresis buffer (0.3 M NaOH and 1 mM Na₂EDTA) and incubated for 40 min at 4°C in the dark. Electrophoresis was performed at 1.1 V/cm² for 20 min. Slides were successively neutralised (0.4 M Tris-HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 µg/ml), washed in PBS, drained and coverslipped.

Evaluation of oxidatively induced DNA damage in PBMCs

The evaluation of oxidatively induced DNA damage was performed as described by Guarnieri *et al.* (23). Cell suspension was supplemented with LMP agarose (1.5% wt/vol) in Tris-acetate-EDTA buffer at pH 7.4 and 37°C and immediately pipetted (10⁴ cells per gel) onto a frosted glass microscope slide (Richardson Supply Co.) precoated with a layer of 1% (wt/vol) NMP agarose similarly prepared in Tris-acetate-EDTA buffer. Cell resistance against oxidatively induced DNA damage was measured by treating the cells with H₂O₂. Two slides with two gels each were prepared for all samples. One slide was treated with H₂O₂ (500 µmol/l in PBS) for 5 min in the dark, and the other slide (in PBS without H₂O₂) was used as a control. Following oxidative treatment, the slides were placed in lysis buffer and processed as previously described (see FPG-sensitive site evaluation). Analysis was performed on 16 samples due to the lack of availability of sufficient PBMCs for two subjects.

Quantification of DNA damage

One hundred comets from the two gels of each slide were electronically captured using an epifluorescence microscope attached to a high sensitivity CCD video camera and to a computer equipped with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). The level of DNA damage was calculated as the percentage of DNA in the tail. For each sample, the percentage of DNA in the tails of control cells (i.e. the cells not treated with H₂O₂ or FPG) was subtracted from that in the tails of the H₂O₂-treated or FPG-treated cells.

Statistical analysis

Statistical analysis was performed using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was used to evaluate the effects of storage as an independent factor and treatment (wild blueberry drink versus placebo drink) and time (before and after each treatment) as dependent factors on FPG-sensitive sites and oxidatively induced DNA damage in PBMCs. Differences between the mean values were evaluated by the least significant difference test. Differences were considered significant at $P \leq 0.05$.

Results

Effects of cryopreservation on background DNA damage and FPG-sensitive sites in PBMCs

ANOVA demonstrated a significant effect of storage on background DNA damage ($P < 0.005$). In particular, cryopreservation of PBMCs increased DNA damage as reported in Table I.

The levels of FPG-sensitive sites in the stored and fresh PBMCs before and after intake of the wild blueberry and placebo drinks are reported in Figure 2. ANOVA, considering storage as independent factor, showed an increase in FPG-sensitive sites in the cryopreserved samples compared with the fresh PBMCs ($P < 0.001$). In particular, the levels of FPG-sensitive sites in the stored samples were higher before and after each dietary treatment compared with the same fresh samples.

A significant time × treatment interaction ($P = 0.044$) was observed in the analysis of the effect of dietary intervention on DNA damage in both the cryopreserved and fresh PBMCs. In fact, a significant reduction in FPG-sensitive sites was found following consumption of the wild blueberry drink (cryopreserved PBMCs: from 22.7 ± 6.1% to 19.1 ± 7.0%, $P = 0.012$; fresh PBMCs: from 12.50 ± 5.6% to 9.6 ± 3.5%, $P = 0.039$) but not after consumption of the placebo drink (cryopreserved PBMCs: from 22.4 ± 5.7% to 20.6 ± 6.8%, $P = 0.650$; fresh PBMCs: 12.0 ± 4.5% to 11.9 ± 4.4%, $P = 0.931$).

Individual levels of DNA damage in the cryopreserved and fresh PBMCs after wild blueberry drink and placebo drink intake are shown in Figure 3. Thirteen out of 18 subjects exhibited a similar trend in the reduction of DNA damage following wild blueberry drink intake independent of storage, while the remaining subjects showed different trends in the stored versus the fresh samples (Figure 3A). With regard to the PBMCs obtained from the placebo intervention, 12 out of 18 individuals showed different trends in DNA damage before and after storage, while the remaining 6 subjects exhibited a similar trend (Figure 3B). These differences were apparently unrelated to the varying storage conditions.

Table I. Background DNA damage in fresh and cryopreserved PBMCs

DNA damage	Before WB	After WB	Before PL	After PL
Background DNA damage (% DNA in tail, EB)				
Fresh PBMCs (N = 18 subjects)	6.4 ± 1.5 ^a	6.6 ± 1.1 ^a	6.3 ± 1.4 ^a	6.1 ± 1.6 ^a
Cryopreserved PBMCs (N = 18 subjects)	12.8 ± 7.4 ^b	12.6 ± 8.5 ^b	14.1 ± 7.9 ^b	11.9 ± 6.5 ^b
Background DNA damage (% DNA in tail, PBS)				
Fresh PBMCs (N = 16 subjects)	6.1 ± 1.1 ^a	6.4 ± 1.1 ^a	6.1 ± 1.3 ^a	6.5 ± 1.4 ^a
Cryopreserved PBMCs (N = 16 subjects)	10.5 ± 5.0 ^b	9.7 ± 4.4 ^b	11.8 ± 6.0 ^b	10.1 ± 4.2 ^b

Data are expressed as mean ± SD. EB, endonuclease buffer; PL, placebo; WB, wild blueberry.

^{a,b}Data with different letters within the same column are significantly different ($P \leq 0.05$).

Effects of cryopreservation on background and H₂O₂-induced DNA damage in PBMCs

ANOVA showed a significant effect of storage on background DNA damage ($P < 0.001$). In particular, cryopreservation of PBMCs increased DNA damage as reported in Table I.

The levels of H₂O₂-induced DNA damage in the stored and fresh PBMCs before and after consumption of the wild blueberry and placebo drinks are reported in Figure 4. ANOVA, considering storage as an independent factor, showed a reduction in oxidatively induced DNA damage in the cryopreserved samples compared with the fresh PBMCs ($P < 0.001$). In particular, the levels of damage in the cryopreserved cells were lower before and after each dietary treatment compared with the same fresh samples.

A significant time \times treatment interaction ($P = 0.043$) was observed in the analysis of the effect of dietary intervention on oxidatively induced DNA damage in the cryopreserved samples compared with the fresh PBMCs. ANOVA of the stored samples did not show a significant reduction in the level of DNA damage following wild blueberry drink intake (from $25.8 \pm 4.6\%$ to $23.9 \pm 4.6\%$, $P = 0.414$), while this effect was evident in the fresh PBMCs (from

$44.7 \pm 7.5\%$ to $36.3 \pm 9.3\%$, $P < 0.001$). In contrast, no effect was observed following placebo drink intake (cryopreserved PBMCs: $23.9 \pm 5.6\%$ to $23.5 \pm 7.1\%$, $P = 0.847$; fresh PBMCs: $46.1 \pm 11.5\%$ to $45.9 \pm 7.1\%$, $P = 0.935$).

Individual levels of H₂O₂-induced DNA damage in the stored and fresh PBMCs after consumption of the wild blueberry and placebo drinks are shown in Figure 5. Ten out of 16 subjects exhibited different trends in the levels of DNA damage between the stored and fresh samples, while the remaining 6 subjects showed a similar trend following wild blueberry drink intake (Figure 5A). With regard to the PBMCs obtained from subjects following placebo drink intake, half of the individuals showed different trends before and after storage, while the others exhibited a similar trend (Figure 5B). These differences were apparently unrelated to the varying storage conditions.

Discussion

The evaluation of DNA damage is very often used as a biomarker of oxidative stress in dietary intervention studies. In fact, the regular intake of food products that are generally rich in bioactive

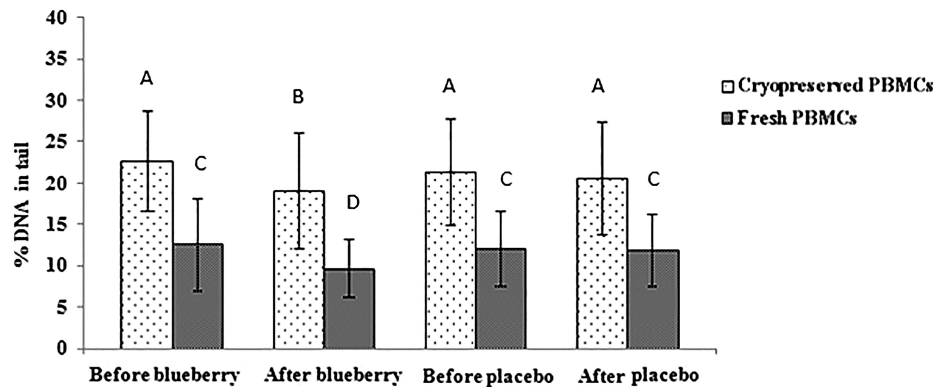


Figure 2. DNA damage, measured as FPG-sensitive sites, in the cryopreserved and fresh PBMCs before and after intake of the wild blueberry and placebo drinks. Data are reported as mean \pm SD ($N = 18$ subjects). (A–D) Data with different letters between and within the bars are significantly different ($P \leq 0.05$).

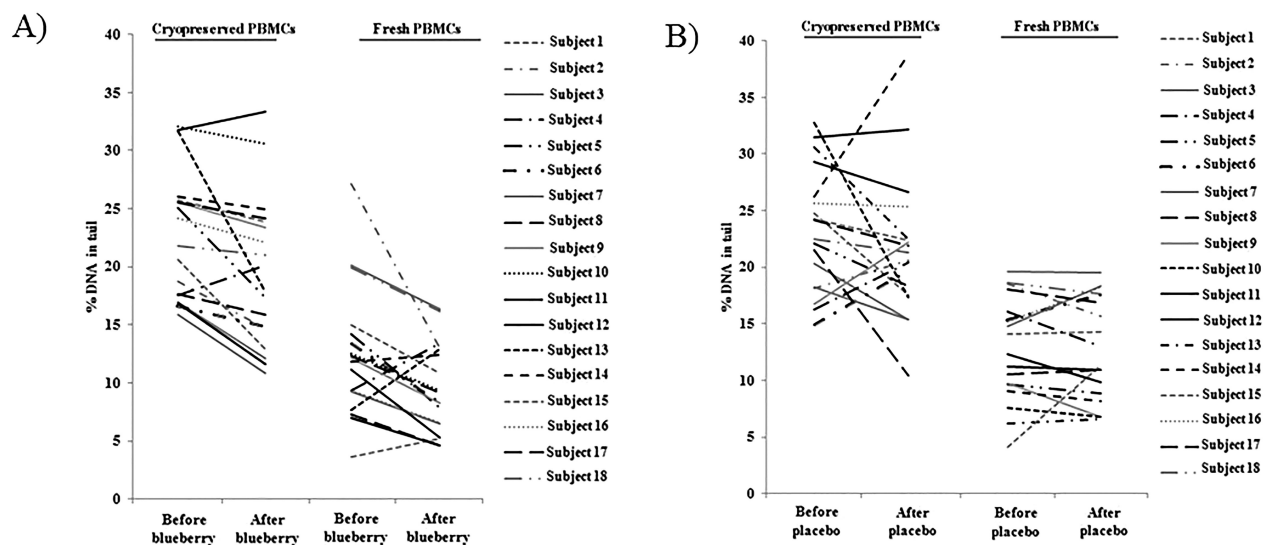


Figure 3. DNA damage, measured as FPG-sensitive sites, in the cryopreserved and fresh PBMCs before and after wild blueberry drink (A) and placebo drink (B) intake in the individual participants ($N = 18$ subjects).

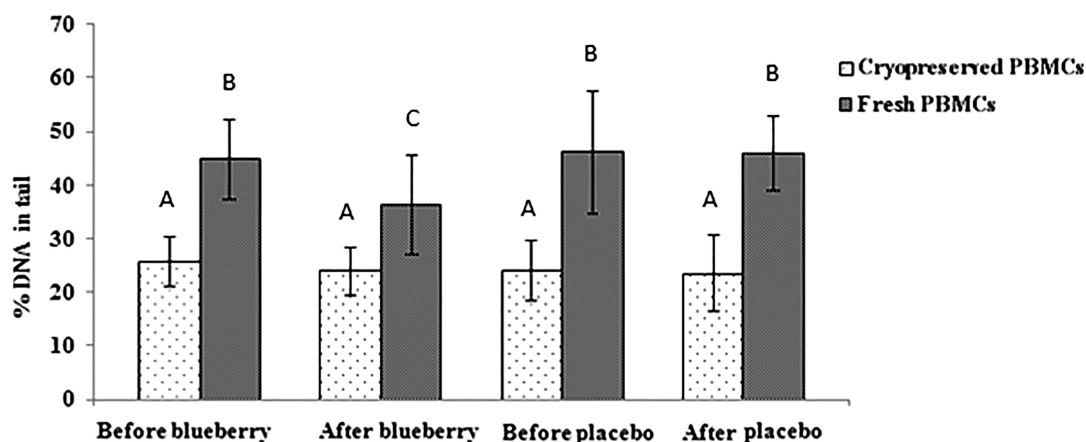


Figure 4. DNA damage, measured as H_2O_2 -induced DNA damage, in the cryopreserved and fresh PBMCs before and after consumption of the wild blueberry and placebo drinks. Data are reported as mean \pm SD ($N = 16$ subjects). (A–C) Data with different letters between and within the bars are significantly different ($P \leq 0.05$).

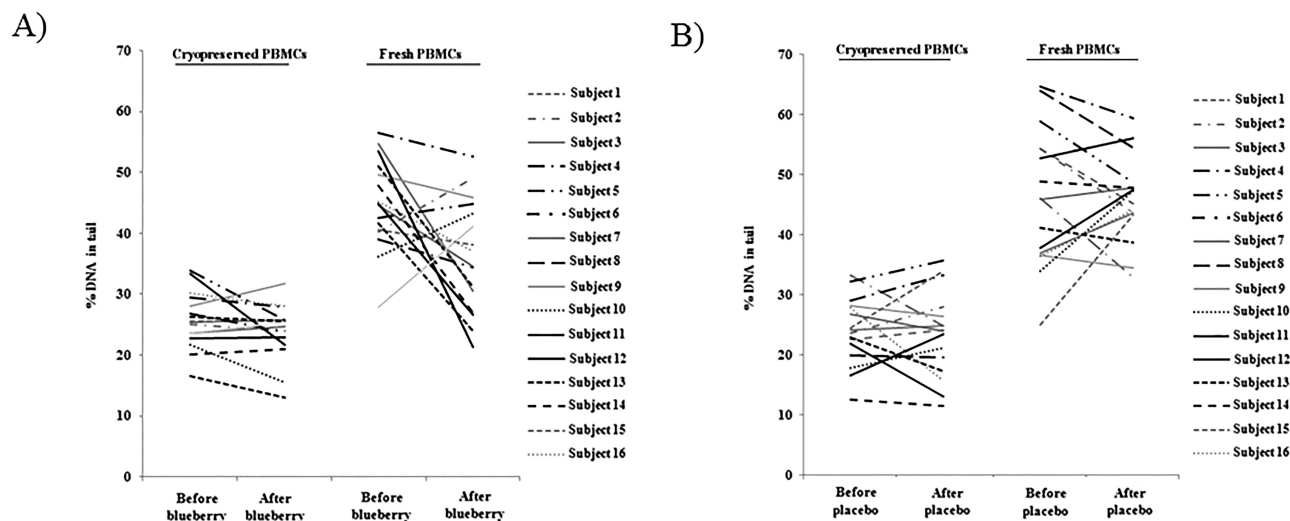


Figure 5. DNA damage, measured as H_2O_2 -induced DNA damage, in the cryopreserved and fresh PBMCs before and after wild blueberry drink (A) and placebo drink (B) intake in the individual participants ($N = 16$ subjects).

compounds with antioxidant properties seems to affect DNA damage to PBMCs as determined by the comet assay (5–10). Data from our laboratory have demonstrated the protective roles of foods such as tomato, broccoli, orange and blueberry in the modulation of endogenous and/or oxidatively induced DNA damage in fresh PBMCs (6,23–25), but the effects of cell storage have not been previously considered. Few studies involving berries are available, and results regarding their DNA-protective effects remain controversial, which may be due to the type of berry studied, the amount consumed, the duration of exposure to the food product/dietary intervention and the biomarker analysed (endogenous versus oxidatively induced DNA damage) (9,26,27). Moreover, variations in the comet assay protocols used (i.e. cell treatment/dilution in agarose, duration of enzyme treatment, duration and pH of alkaline treatment, electrophoresis conditions and slide scoring) can significantly affect results, and this has been documented in several validation studies (28–33).

Another important but sometimes neglected variable that should be carefully considered is the impact of cell cryopreservation on DNA damage. The majority of data on DNA damage is derived from

analyses performed on cryopreserved cells, but in some cases, there is no mention of the storage process (e.g. method of cryopreservation, duration of storage), and very few studies have used fresh samples. To our knowledge, this is the first study comparing levels of DNA damage, which were measured as FPG-sensitive sites and H_2O_2 -induced DNA damage, in fresh versus cryopreserved PBMCs obtained from individuals participating in a dietary intervention study. We demonstrated that sample cryopreservation significantly increased (up to 140%) DNA damage compared with that observed in fresh samples. Increased damage was evident in control cells (without FPG or H_2O_2 treatment) and in FPG-treated cells. These results are in accordance with studies showing an increase in DNA damage after a freeze-thaw process (34,35), but they are in contrast with other studies reporting that cryopreservation does not affect DNA damage (17,36). It has been shown that DNA damage of above 10% in PBMCs may indicate that some DNA breakage has occurred during storage (36). Our results are in accordance with this observation because the levels of background DNA damage were $>10\%$. Several mechanisms could be responsible for the increased damage that occurs during

cryopreservation, including mechanical injury due to ice crystal formation (37). Al-Salmani *et al.* (38) have documented that the storage of large volumes (~5 ml) of blood for up to 2 months leads to an increase in DNA damage. However, the authors have reported that DNA damage could be minimised by preparing small aliquots (200 µl) of blood or by adding cryopreservatives to larger sample volumes (36,38). To store isolated PBMCs, a cryopreservative is needed. In the present study, small aliquots of PBMCs (1 ml) were prepared, and DMSO was added to the cells to prevent the formation of ice crystals and to protect them from damage. Moreover, a freezing container (Mr. Frosty, Thermo Scientific, USA) with isopropyl alcohol was used to control the rate of cooling (close to $-1^{\circ}\text{C}/\text{min}$) to -80°C . Despite these controlled experimental conditions, we found increased levels of DNA damage; thus, other factors, including oxidative stress, altered physical properties of cellular structures, osmotic injury, cytokine and protein secretion or an extended duration of storage, may have contributed to this finding (37).

The impact of cell cryopreservation on DNA damage is particularly important when analysing results from dietary intervention studies. In the present study, we observed that PBMC cryopreservation significantly increased the levels of FPG-sensitive sites compared to the fresh samples but reductions in DNA damage were observed following wild blueberry drink consumption in both the fresh and stored PBMCs (-16.6% versus -14.6% , respectively, $P = 0.85$). These results suggest that FPG-sensitive sites may be sufficiently reliable for the evaluation of DNA damage in both fresh and cryopreserved PBMCs collected from individuals participating in dietary intervention studies. However, it is noteworthy that a potential risk of obtaining different results following cell storage cannot be excluded. In fact, we found high individual variability in DNA damage before and after the dietary treatments in the fresh compared with the cryopreserved samples (Figure 3).

In the literature, few studies have reported the role of berries in the modulation of endogenous DNA damage, and results have been generally obtained using stored samples. Bub *et al.* (39) have observed a reduction in endogenous DNA damage in PBMCs (information on storage was not reported) following 2-week consumption of polyphenol-rich juice (330 ml/day, obtained through a mix of berries and other fruits). In contrast, Duthie *et al.* (27) have documented that 2-week of cranberry juice intake (750 ml) does not modulate endogenous DNA damage in cryopreserved PBMCs. Similar results have also been observed by Møller *et al.* (9) after 3-week consumption of blackcurrant juice (1000 ml/day).

With regard to oxidatively induced DNA damage, we found a significantly different response between the cryopreserved and fresh cells. Although cryopreservation produced higher background DNA damage with respect to that found in fresh PBMCs, an increased resistance to H_2O_2 -induced DNA damage was documented. This improvement in cell defence masked the protective effects of the blueberry drink treatment demonstrated in the fresh samples. This increased response of the cryopreserved PBMCs may have been due to the activation of protective mechanisms, such as the expression of antioxidant enzymes (i.e. catalase, superoxide dismutase or glutathione peroxidase) (40), but this hypothesis should be confirmed. In contrast with our observation, Duthie *et al.* (17) have found that fresh and frozen cells respond almost identically to H_2O_2 treatment, while Visvardis *et al.* (41) have documented a significant increase in H_2O_2 -induced DNA damage in cryopreserved lymphocytes.

Limited results are available with regard to the modulation of *ex vivo* H_2O_2 -induced DNA damage following berry interventions. For example, in a previous study, we have documented that one portion

of blueberries (300 g) is able to increase the resistance of DNA to H_2O_2 in fresh PBMCs (25). The same results have been found by Wilms *et al.* (26) in fresh PBMCs following 4-week consumption of blueberry/apple juice (1000 ml/day), while no effect was observed in cryopreserved PBMCs after cranberry juice consumption (27).

In conclusion, our results suggest that FPG-sensitive sites and oxidatively induced DNA damage can be significantly modified following the long-term storage of samples obtained from individuals participating in dietary intervention. Despite the overall significant increase in FPG-sensitive sites that was demonstrated in this study, the comparable protective effect of the dietary treatment seems to suggest that this biomarker is reliable for the assessment of both cryopreserved and fresh samples. In contrast, dietary intervention failed to affect H_2O_2 -induced DNA damage in the cryopreserved PBMCs. Further research is needed to clarify the reliability of the assessment of H_2O_2 -induced DNA damage following cell storage.

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P.R. and M.P. designed the study and obtained funds. C.D.B., D.F., and C.L. performed the experiment and analysed the data. All authors contributed to the writing and proofreading of this manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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