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Abstract

Due to the great number of genes involved in DNA repair and the interactions among the pathways responsible for the repair of different types of DNA damage, there is an increasing need for simple and reliable approaches to phenotypically assess DNA repair capacity (DRC). The use of peripheral blood mononuclear cells (PBMCs) in DRC assays is particularly useful for human monitoring studies. However, in such studies it is not always possible to collect and process samples on the same day as the blood is taken.

We performed a genotype–phenotype correlation study on DRC on 225 healthy subjects. Due to the large number of blood samples to be processed, PBMCs were either isolated and cryopreserved on the same day of blood collection (day 1) or on the following day after 24 h blood storage at room temperature (day 2-RT). Samples processed in different days showed a significant difference in the DRC evaluated as 8-oxoguanine glycosylase activity (OGG assay) in cell extracts ($p < 0.0001$) and as benzo[a]pyrene diol epoxide (BPDE)-induced damage repair by the comet assay ($p = 0.05$). No apparent effect of the blood storage conditions on the outcome of γ-ray induced H2AX phosphorylation assay was reported.

These results prompted us to further analyze the effects of blood storage conditions by performing a validation study. Three blood samples were simultaneously taken from ten healthy donors, PBMCs were isolated and cryopreserved as follows: immediately after blood collection (day 1); on the following day, after blood storage at RT (day 2-RT); or after blood storage at 4 °C (day 2-4 °C). DRC was then evaluated using phenotypic assays. The γ-ray induced H2AX phosphorylation assay has been confirmed as the only assay that showed good reproducibility independently of the blood storage conditions. The measurement of OGG assay was most affected by the blood storage conditions.

Abbreviations

- PBMCs, peripheral blood mononuclear cells;
- DSBR, double-strand break repair;
- IR, ionizing radiation;
- NER, nucleotide excision repair;
- BER, base excision repair;
- OGG1, 8-oxoguanine glycosylase;
- DRC, DNA repair capacity;
- FPG, formamidopyrimidine DNA glycosylase;
- BPDE, benzo[a]pyrene diol epoxide;
- RT, room temperature

1. Introduction

The repair of DNA damage is essential for maintenance of genome stability and a defective DNA repair is strongly associated with the risk of cancer and other human diseases [1]. At least four main repair pathways operate in the removal of DNA lesions, which partly overlap each other: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and DNA double-strand break repair (DSBR) [1] and [2]. To date, several *in vitro* assays have been developed to evaluate DNA repair capacity (DRC). These methods vary from measurements of the removal of induced DNA damage on *in vitro* challenged

cells as in the comet assay [3], to assays based on the induction of histone modifications (such as the H2AX phosphorylation assay) [4] or to assays measuring the DRC in cell extracts [5] and [6].

Peripheral blood mononuclear cells (PBMCs) provide a convenient and readily available source of human material and are routinely used experimentally to assess DNA damage and repair. Logistically, samples may require to be stored and PBMC isolation is a labor-intensive and time-consuming step based on density gradient centrifugation, and in large studies it is not always possible to take the blood, collect and process the samples on the same day. However, how blood storage, cell isolation and freezing can affect PBMCs used in human biomonitoring studies remain to be established. Previous results showed that DNA strand breaks in human lymphocytes are greatly increased by the concurrent lysis of whole blood, suggesting that careful preparation of PBMCs is of paramount importance to assess DNA damage and repair [7]. Lymphocytes undergo apoptosis during storage and this loss of viability is accelerated by increasing both temperature and storage time. Phytoemoagglutinin (PHA) stimulated whole blood stored at both 4 °C and 20 °C for 48 h showed lower percentage of apoptosis compared with storage at 37 °C and the mitotic index values demonstrate that the best ability to proliferate was preserved in samples stored in the presence of PHA and at 4 °C [8]. Results of studies on the analysis of leukocyte cytokine production from blood stored at 4 °C for 24 h showed less immunomodulatory changes than blood kept at room temperature [9].

We collected blood from 225 healthy subjects with the aim to perform a large genotype–phenotype correlation study on DRC and SNPs in DNA repair genes [10]. Due to the large number of blood samples collected daily, some PBMC samples could not be processed on the same day of blood collection. When different DRC assays were performed on these samples, a statistically significant difference between the results obtained with some DRC assay on samples processed on the same day of blood collection (day 1) and those processed on the following day, after 24 h storing of blood at room temperature (day 2-RT) was observed.

Therefore, we decided to better investigate the effects of storage conditions of blood samples on the outcome of various DRC assays such as aphidicolin-block NER comet assay [11] and [12], H2AX phosphorylation to measure DSBR [4] and [13] and 8-oxoguanine glycosylase activity assay (OGG assay) to monitor the activity of BER DNA glycosylases [5] and [14]. Blood was collected from 10 healthy donors and stored under different conditions before PBMC separation and cryopreservation: up to 4 h at room temperature (fresh blood), 24 h at room temperature or 24 h at 4 °C. The blood storage conditions were confirmed to affect DRC, particularly when measured in cell extracts.

2. Materials and methods

2.1. Samples and PBMC isolation

The genotype–phenotype study on DRC and DNA repair gene polymorphisms was conducted on 225 healthy subjects (149 females, 76 males; mean age years ± S.D. 24.2 ± 6.2, range 18.4–48.4). Thirty milliliters of heparinized venous blood were collected from all subjects. PBMCs were separated within 4 h from blood collection (day 1) or after 24 h blood storage at room temperature (day 2-RT) (see below for PBMC separation procedure).

For the validation study, 10 healthy subjects (5 males, 5 females; mean age years ± S.D. 32.4 ± 7.4, range 25–45) were included. Ten milliliters of heparinized venous blood were collected in three different vacuum blood collection tubes from each subject. The blood samples were processed in three different conditions modifying both the time elapsed between the collection and the PBMC separation/cryopreservation (day 1: all the process in the same day of sample collection; day 2: PBMCs separation and cryopreservation 24 h after the sample collection), and the storage conditions of the blood samples processed on day 2 (at room temperature or at 4 °C).

PBMCs were separated by centrifugation with FicollPaque PLUS (GE Healthcare, Milan, Italy) at 400 × *g* for 30 min at room temperature. After two washes in Washing Medium (RPMI 1640, 1% FBS, 25 mM EDTA), PBMCs were prepared for cryopreservation. They were suspended at 10×10^6 cells/ml in freezing medium (RPMI 1640, 50% FBS, 10% DMSO), aliquoted in cryovials and frozen overnight at a −1 °C/min rate in isopropyl alcohol up to −80 °C (Mr. Frosty containers, Nalgene, Roskilde, Denmark). Cryovials were then transferred in liquid nitrogen for long storage. All PBMCs used in the present study were cryopreserved before use.

2.2. Cell extracts and DNA substrate preparation for OGG1 activity assay

Cell extracts were prepared as described by Paz-Elizur et al. [5]. Briefly, a hypotonic buffer solution containing Tris–HCl 50 mM, pH 7.1, EDTA 1 mM, spermidine 0.5 mM, spermine 0.1 mM, dithiothreitol 0.5 mM and a protease inhibitor cocktail (Roche, Molecular Biochemicals, Mannheim, Germany) was added to isolated PBMCs, at concentration of 40,000 cells/µl. The PBMCs were incubated on ice for 30 min, then they were frozen in liquid nitrogen and stored at −80 °C overnight. Frozen PBMCs were lysed by quickly thawing them in a water bath at 30 °C and then incubated on ice with KCl 220 mM for 30 min. Extracts were subsequently cleared by centrifugation at 11,000 × *g* for 15 min at 4 °C. After glycerol addition at a final concentration of 10%, the samples were stored at −80 °C. HPLC purified 30-mer oligonucleotides (Thermo Fisher Scientific GmbH, Ulm, Germany) containing a single 8-oxoG and 3' end labeled with 6-carboxyfluorescein (6-FAM) (5' GCG TAG AGC ATT GoxoGT ATC CGA GCG TCA GCG 3') were annealed to their complementary strands by heating them at 90 °C for 10 min and by a slow cooling down at room temperature (8-OH-G:C substrate). Duplex DNA homogeneity was checked by non-denaturing 10% polyacrylamide gel electrophoresis (PAGE). Fluorescent bands were visualized by Typhoon 9200 Gel Imager (GE Healthcare Bioscience AP, Uppsala, SE) and quantitated by using the public domain NIH Image software (http://rsb.info.nih.gov.offcampus.dam.unito.it/ij/).

2.3. OGG activity assay

The DNA–glycosylase activity in cell extracts was determined essentially as previously described [14]. The 8-OH-G:C substrate (5 nM) was incubated with 10 µg of protein extracts in 25 µl of 20 mM Tris–HCl pH 8.0, 80 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA buffer at 37 °C for 1 h, followed by alkaline treatment (80 mM NaOH for 2 min), to completely remove residual abasic sites produced by OGG reaction. DNA products were separated by 20% denaturing PAGE at 500 V for 2.5 h. Fluorescent bands corresponding to the intact oligonucleotide and to the cleaved product were analyzed by Typhoon scanner and quantified by using the public domain NIH *Image J* software (http://rsb.info.nih.gov.offcampus.dam.unito.it/ij/). Data were analyzed by Kaleidagraph software (Synergy, Reading, PA, USA).

2.4. mRNA expression

mRNA expression analysis was performed directly from aliquots of PBMCs stored in liquid nitrogen. Five million cells were pelleted, and total RNA was isolated using a column affinity procedure (RNeasy Mini Kit, QIAGEN, Valencia, CA) according to manufacturer's dispositions. A DNA nuclease treatment step was included to prevent genomic DNA carry-over. After accurate quantitation of RNA by a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), 1 µg total RNA was reverse transcribed to single stranded cDNA by High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, adding 1 U/µl final concentration of RNAse inhibitor (Applied Biosystems). A validation step to test the linearity of PCR efficiency over a broad range of initial template concentrations was performed on the gene-expression pre-made assays used in the study, and plate to plate consistency was tested including replicated controls in each Real Time-PCR (RT-PCR) plate. Primers and probes for the target gene *OGG1* were purchased as pre-made assays (TaqMan Gene Expression Assay) from Applied Biosystems, and analyses carried out according to the producer's instructions. As reference genes, Glucose 6-Phosphate Dehydrogenase (*G6PD*) and Abelson (*ABL*) genes (TaqMan Gene Expression Assay, Applied

Biosystems) were used. Real Time RT-PCR analyses were carried out on a ABI Prism 7900 HT Fast Real Time PCR System (Applied Biosystems), adding 2 µl of cDNA to the proper amount of Genotyping Master Mix (Applied Biosystems) and TaqMan Gene Expression Assay (Applied Biosystems) in a total 15 µl volume reaction. Three replicates for each sample were analyzed. A no-template control (NTC), a no-reverse transcription control (RT-), as well as a positive control to test for reproducibility were included in all the RT-PCR plates for each assay. $RQ^{\Delta\Delta Ct}$ was defined as the value $2^{-\Delta Ct}$, where ΔCt = *Ct* target gene − *Ct* reference gene. This values were used to show *OGG1* gene expression in the healthy population of 225 subjects. When the same reaction conditions were maintained in mRNA expression analyses performed on *OGG1, ABL* and *G6PD* genes (validation study on 10 subjects, different blood storage conditions), we compared raw *Ct* values from different RT-PCR experiments. Indeed, the absolute *Ct* value comparison is only meaningful in this situation, whereas it is not feasible if different instruments, reagents, primers and probes, or reaction volumes are employed to produce the different *Cts* [15].

2.5. H2AX phosphorylation assay

PBMCs were thawed and allowed to recover overnight in complete RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (heat inactivated at 56 °C for 30 min) (Invitrogen). Cells were γ-irradiated (2 Gy) using a 6 MV accelerator (Elekta, Stockholm, Sweden) at 2 Gy/min. They were kept on ice for 1 h (the time needed to reach the laboratories), and then returned to the incubator and harvested at 1 h and 3 h. Cells were stained with anti-phospho-histone H2AX (Ser-139) (Upstate Biotechnology, Charlottesville, VA, USA) according to Olive et al. [16], with minor modifications [4].

Approximately 400,000 irradiated cells were collected, fixed in cold 70% ethanol and stored at −20 °C for up to 2 weeks before analysis. Cells were washed in Tris-buffered saline pH 7.4 (TBS) and then rehydrated for 10 min at 4 °C in TBS containing 4% FBS and 0.1% Triton X-100 (TST) (Sigma–Aldrich Co., St Louis, MO, USA) prior to staining with anti-γH2AX mAb (Upstate Biotechnology) diluted at 1:250 in TST, and incubated for 2 h at 37 °C. After two washes in TBS, they were suspended in FITC-conjugated goat anti-mouse IgG1 (BD PharMingen, Becton Dickinson & Co., Franklin Lakes, NJ, USA) diluted at 1:50 in TST as a secondary antibody, and shaken for 1 h at room temperature in the dark. A minimum of 10,000 stained cells were acquired on a FACScan (Becton Dickinson & Co.) and analyzed with the CellQuest software. We maintained the same cytofluorimeter setting for staining on different days (SSC: voltage 402, AmpGain 1, Mode lin., FL1: voltage 490, AmpGain 1, Mode log). In order to normalize the results, aliquots of PBMCs derived from the same subject were used at each irradiation and staining step as control.

The data corresponding to the amount of H2AX phosphorylation at 1 h are expressed as ratio of the median of fluorescence of γH2AX in the sample, relative to the median of fluorescence expressed by the control. The data corresponding to the dephosphorylation of H2AX after 3 h of recovery are expressed as percentage of the median of γH2AX fluorescence at 3 h with respect to the median of γH2AX fluorescence shown at 1 h by the same sample.

2.6. Aphidicolin-block NER comet assay

Cryopreserved PBMCs were thawed quickly in a water bath at 37 °C and suspended in 5 ml cold medium containing 50% FBS, 49% RPMI 1640 and 1% dextrose. Treatment was performed as previously described [11] and [12], with minor modification. Cells were spun down by centrifugation (10 min at 400 × *g*) at 4 °C, and 5×10^6 viable cells were suspended in 5 ml F10 medium (Invitrogen, Paysley, UK) containing 10% FBS and 2.5 µg/ml phytohaemoagglutinin (PHA) (Sigma–Aldrich Co, St Louis, MO). Twenty-four hours later samples were centrifuged (10 min at 400 × *g*) at 4 °C, and cell pellets were suspended in 5 ml of F10 medium without FBS containing 2.5 µg/ml PHA; cells were then divided in 3 tubes (A, B, C for each sample) and treated in the following ways: (A) 2.5 µg/ml aphidicolin (APC, Sigma–Aldrich Co), 30 min at 37 °C, 5% CO_2; (B) 0.5 µM benzo[a]pyrene diol epoxide (BPDE; NCI Chemical Carcinogen Reference Standards Repository, Midwest Research Institute, Kansas City, MO, USA), 2 h at 37 °C, 5% CO_2; (C) pretreatment with

2.5 μg/ml APC (30 min) followed by 0.5 μM BPDE (2 h). At the end of the treatment cells were centrifuged and pelleted cells were processed for comet assay.

The comet assay was performed as previously described [11] and [12]. After treatment, cells were mixed with low-melting-point agarose (0.75%; Sigma–Aldrich, St Louis, MO) and layered on 85 × 100 mm GelBond films (Lonza, Basel, Switzerland). Each GelBond film comprised eight 19 × 23 mm agarose gels. The GelBond films were immersed in lysis solution (2.5 M NaCl, 0.1 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1.5 h at 4 °C, then placed in an electrophoresis tank for 40 min submerged by electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, approximately pH 13), and finally electrophoresed at 30 V (0.8 V/cm) and 300 mA for 20 min. After neutralization with 0.4 M Tris–HCl, pH 7.5, gels were fixed in ethanol and dried at room temperature. For scoring, slides were stained with YOYO-1 iodide (1 mM solution in DMSO, diluted 1:250 in PBS; Life Technologies Italia, Monza, Italy) and nuclei were visualized by a Leica fluorescence microscope at 40× magnification. Two gels of 50 nuclei for each sample condition were scored with Comet IV software (Perceptive Instrument, Suffolk, UK). Median fluorescence of tail intensity (% Tail DNA) of 100 nuclei was used as a measure of DNA damage. For each electrophoresis, human K562 erythroleukemia cell line samples were included as internal standard and used for results normalization as previously described [12]. For each subject, DRC was calculated as follows: % Tail $DNA_{APC+BPDE}$ − % Tail DNA_{BPDE} − % Tail DNA_{APC}.

2.7. Statistical analysis

Data were presented as means and standard deviation. We tested for variation across the three different conditions (day 1, day 2-RT and day 2-4 °C) by using the Wilcoxon Rank Sum test, Wilcoxon Signed Rank test and Kruskal–Wallis test as appropriate. Pearson coefficient was used for correlation assessment. All analyses were conducted using SAS V 9.2.

3. Results and discussion

3.1. Effect of blood storage on DNA repair assays and mRNA expression in 225 healthy subjects

Within a genotype–phenotype association study between DRC and SNPs in DNA repair genes [10], blood samples from 225 healthy subjects were collected. PBMCs from 138 subjects were processed (*i.e.* isolated and cryopreserved) the same day of blood collection (day 1), while in the case of the remaining 87 subjects, the PBMC processing was delayed by 24 h (day 2-RT). The following DRC assays were performed on all the samples: OGG activity assay, H2AX phosphorylation assay, and aphidicolin-block NER comet assay. As shown in Table 1, a statistically significant difference was observed when comparing the results of the NER and OGG activity assays performed on the two sets of samples, whereas the outcome of the H2AX phosphorylation assay was unaffected by the storage conditions. In particular, the DRC evaluated by NER comet assay was lower in “day 2-RT” than in “day 1” samples (p-value = 0.05) and OGG activity was higher in “day 2-RT” than in “day 1” samples (p-value = < 0.0001). DRC evaluated by H2AX phosphorylation assay was comparable in the two conditions. *OGG1* gene expression was monitored by RT-PCR on the same samples. OGG1 transcript levels were lower in “day 2-RT” samples than in “day 1” samples (p-value < 0.0001). OGG1 activity and *OGG1* gene expression levels, presented a significant inverse correlation, although weak (Pearson coefficient $r = -0.22$, $p = 0.024$; data not shown). This inverse correlation between OGG activity and expression supports the view that OGG1 mRNA levels are not a marker of 8-oxoguanine DNA repair activity [18].

Table 1. Differences in DRC evaluated with different assays in 225 healthy subjects in PBMCs isolated and cryopreserved on the same day of blood collection (day 1) or after 24 h blood storage at room temperature (day 2-RT). p-Values were obtained with Kruskal–Wallis analysis. Sample numbers do not sum up to the total due to limitation of cells or assay failure.

	OGG activity (fmoles/µg)		Comet NER (DRC)		H2AX 1 h phosphorylation (AU)		H2AX 3 h dephosphorylation (%)		OGG1 expression ($RQ^{\Delta\Delta Ct}$)[a]	
	Day 1 (*n* = 104)	**Day 2-RT (*n* = 64)**	**Day 1 (*n* = 122)**	**Day 2-RT (*n* = 54)**	**Day 1 (*n* = 82)**	**Day 2-RT (*n* = 36)**	**Day 1 (*n* = 82)**	**Day 2-RT (*n* = 36)**	**Day 1 (*n* = 126)**	**Day 2-RT (*n* = 83)**
Mean	2.05	2.42	7.38	5.84	18.96	18.46	0.90	0.90	2.67	1.57
S.D.	0.46	0.54	4.99	3.95	11.57	9.25	0.16	0.15	1.29	0.90
Min	0.86	1.36	0.66	0.31	0.00	0.28	0.59	0.61	1.40	0.46
Max	3.35	3.52	26.15	25.58	56.79	38.75	1.36	1.14	9.77	4.80
KW *p*-value	<0.0001		0.05		0.91		0.93		<0.0001	

a

$RQ^{\Delta\Delta Ct}$ was defined as the value $2^{-\Delta Ct}$, where ΔCt = *Ct* target gene − *Ct* reference gene (reference gene: ABL).

Previous reports [7] and [19] of effects on DNA damage and repair capacity of blood storage and PBMC cryopreservation together with our observation of statistically significant differences in the measurements of DRC by various assays after blood storage at room temperature, prompted us to perform a validation study where the effect of blood storage conditions on DRC as measured by different assays was specifically addressed. We collected blood sample from 10 healthy volunteers and PBMCs were isolated and cryopreserved the same day of blood collection or the following day, after 24 h blood storage at room temperature or at 4 °C. DRC was then analyzed with different DNA repair assays. Two types of assays were conducted: measurement of basal DNA repair activity in cell extracts (OGG assay) and measurement of DNA repair in stimulated PBMCs after exposure to a DNA damaging agent (aphidicolin-block NER comet assay and H2AX phosphorylation assay after BPDE and γ-ray exposure, respectively).

3.2. Effect of blood storage on OGG activity

As shown in Fig. 1, a trend toward higher mean values was recorded with lower OGG activity in PBMCs from “day 1” blood (mean ± S.D. 1.42 ± 0.22 fmoles/µg total proteins), increased levels from blood stored 24 h at room temperature (“day 2-RT” mean ± S.D. 1.50 ± 0.35 fmoles/µg total proteins) and the highest levels at 4 °C (“day 2-4 °C” mean ± S.D. 1.79 ± 0.46 fmoles/µg total proteins), but the differences were not statistically significant (*p*-value = 0.11 and *p*-value = 0.91 respectively). This assay was previously shown to be robust and reproducible when used on different PBMC extracts from the same blood sample (intra-assay CV = 8%) [14]. The lack of correlation observed in this study between OGG activity values of PBMC extracts from blood samples of the same subject but stored under different conditions indicates that the differences are due to the effects of blood storage.

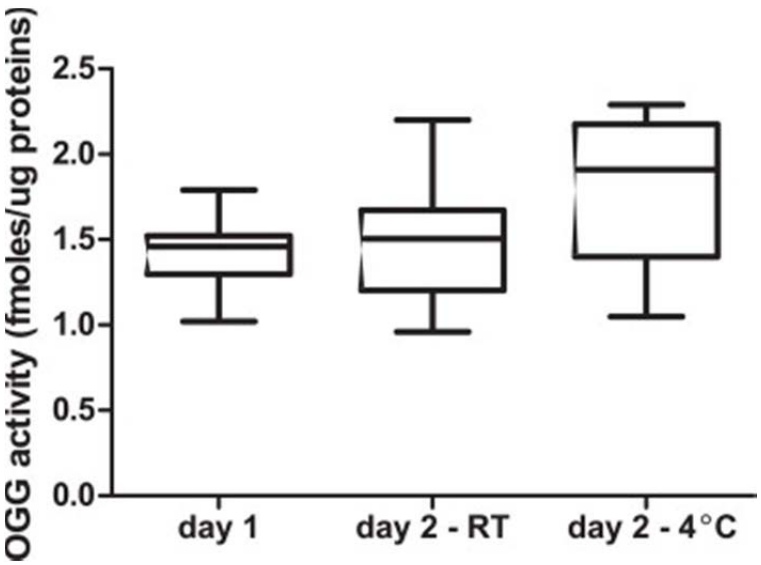

Fig. 1.

Effect of different blood storage conditions on OGG activity on 10 healthy subjects.

It is well known that biochemical and physical changes occur in the blood cell population as a function of storage and oxidative damage is one of the major factors contributing to the development of "storage lesions" [20]. Changes in various enzymatic activities have been observed in blood as a function of storage [21] and OGG1 is particularly sensitive to the oxidative environment [22] and [27]. Therefore our observation discourages to perform OGG activity assay on extracts obtained from PBMCs isolated from blood stored for 24 h after collection. It is strongly suggested to perform the assay only on PBMC samples isolated from fresh blood.

3.3. Effect of blood storage on mRNA expression analysis

Owing to the observation of a remarkable difference in *OGG1* expression in the 225 subjects previously analyzed (Table 1), mRNA expression was assessed by RT-PCR using primers and probes for the target gene *OGG1* and for the reference genes *G6PD* and *ABL*. As our goal was to detect the possible effect of different blood storage conditions prior to PBMC isolation and cryopreservation on mRNA expression, we analyzed and compared the raw *Ct* values for the three genes. We observed statistically significant differences between "day 1" and "day 2-RT" samples in *G6PD* (*p*-value = 0.002) and *OGG1* (*p*-values = 0.002) *Ct* values; between "day 1" and "day 2-4 °C" in all the genes *Ct* values (*ABL p*-value = 0.01; *G6PD p*-value = 0.004; *OGG1 p*-value = 0.006) (Fig. 2). Despite these differences, "day 1" *versus* "day 2-RT" samples showed a strong correlation (*ABL r* = 0.677, *p*-value = 0.03; *G6PD r* = 0.866, *p*-value = 0.001; *OGG1 r* = 0.806, *p*-value = 0.005).

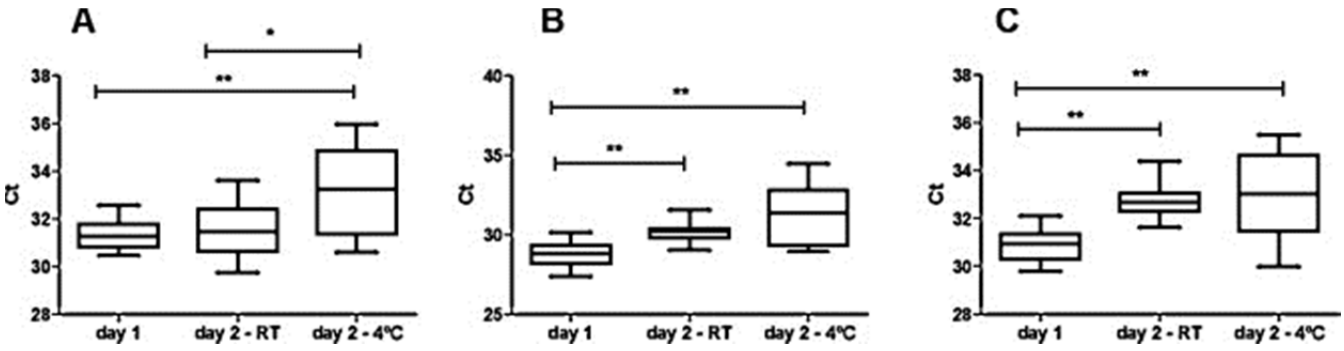

Fig. 2.

Effect of different blood storage conditions on mRNA expression on 10 healthy subjects. (A) ABL; (B) G6PD; (C) OGG1. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

Although a strong correlation was observed between the values obtained on "day 1" and "day 2-RT" samples, the levels of transcripts changed significantly depending on the storage conditions (Wilcoxon Signed Rank test analysis), suggesting that quantitative mRNA analysis should be done on samples isolated under the same conditions. Moreover, mRNA expression turned out not to be applicable to PBMCs isolated and cryopreserved after 24 h blood storage at 4 °C. Our findings are in agreement with the analysis of a large set of gene expression data that showed dramatic changes in relative gene expression for a number of cytokines, chemokines, and transcription factors in blood that has been stored at room temperature for greater than 1 h *versus* blood samples that have been lysed immediately post-collection [23].

3.4. Effect of blood storage on H2AX phosphorylation assay

Basal H2AX phosphorylation was evaluated in untreated PBMCs: we observed a slight, but not statistically significant, increase of H2AX phosphorylation after 24 h of blood storage, both at RT (p-value = 0.08) and at 4 °C (p-value = 0.21) (Fig. 3A). Moreover, we found that H2AX phosphorylation at 1 h after γ-radiation exposure was lower in PBMCs from "day 2-4 °C" samples (mean ± S.D. 15.98 ± 5.36 median of fluorescence) than in other conditions (mean ± S.D. 16.21 ± 3.21 "day 1"; p-value = 0.64; 16.25 ± 3.41 "day 2-RT"; p-value = 0.91), but the difference was not statistically significant (Fig. 3B). Correlation analysis showed significant results for H2AX phosphorylation at 1 h for "day 1" *versus* "day 2-RT" samples (r = 0.892, p-value = 0.003). Similar results were obtained when the percentage of H2AX dephosphorylation observed under different conditions was evaluated ("day 1" *versus* "day 2-RT", p-value = 0.05; "day 1" *versus* "day 2-4 °C", p-value = 0.74) (Fig. 3C). Correlation results were statistically significant for the percentage of H2AX dephosphorylation for "day 1" *versus* "day 2-RT" samples (r = 0.833, p-value = 0.01).

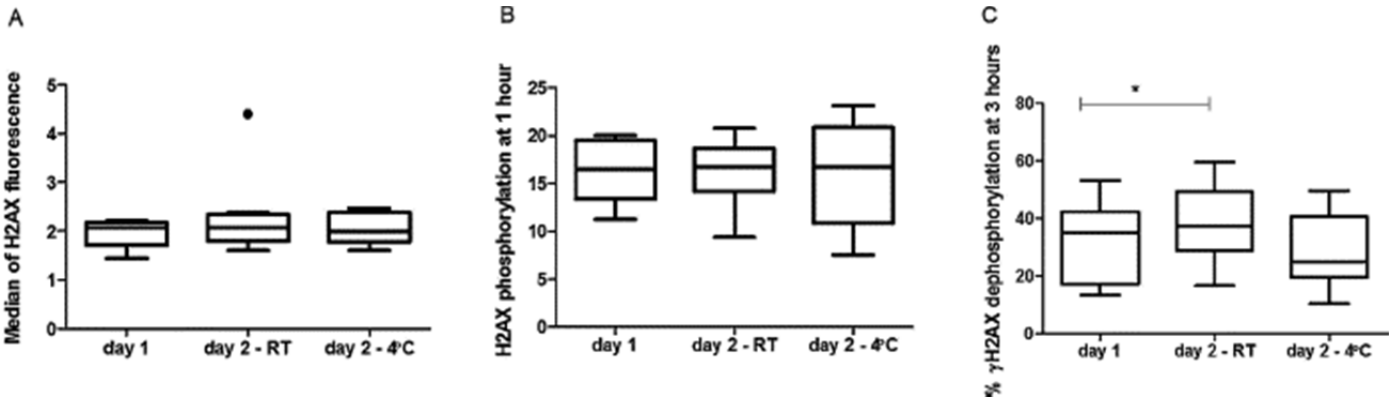

Fig. 3.

Effect of different blood storage conditions on 10 healthy subjects on: (A) background H2AX phosphorylation; (B) H2AX phosphorylation after 1 h from γ-irradiation; (C) % H2AX dephosphorylation after 3 h from γ-irradiation. *: $p \leq 0.05$

These results suggested that DNA damage recognition after γ radiation can be reproducibly evaluated through this approach irrespective of the blood storage/PBMC isolation conditions, as in part already observed on the 225 healthy subjects. However, when we looked at the percentage of H2AX dephosphorylation after 3 h, we observed a difference between "day 1" and "day 2-RT" samples (p-value = 0.05). Moreover, we observed a correlation (r = 0.833, p-value = 0.01) between "day 1" and "day 2-RT" samples, again suggesting that H2AX assay can be performed with reliable results in both conditions. Probably, this behavior is due to the fact that the γH2AX assay implies an overnight recovery of PBMCs in complete RPMI after thawing. It can be speculated that during the recovery time, cells repair the possible DNA alterations caused by the different storage conditions and reset their DRC. When stability of ATM protein, the main kinase involved in H2AX phosphorylation following a DNA double-strand break, was

analyzed in heparinized whole blood, it was found that ATM protein concentrations decreased by 24–15% when blood was stored at room temperature for 1 day [24]. Based on our results, this moderate decrease in ATM protein concentrations does not impact on protein activity, at least on its H2AX target. Stability of ATM protein in frozen PBMCs stored at −70 °C for a 4-week period was previously demonstrated [24].

3.5. Effect of blood storage on NER comet assay

BPDE-induced DRC was affected by the blood storage conditions. In fact, while "day 1" and "day 2-4 °C" samples showed comparable NER activity (mean ± S.D. 3.13 ± 1.48 and 3.38 ± 2.06 DRC respectively), DRC of "day 2-RT" samples was higher (mean ± S.D. 4.22 ± 1.56), though the difference not statistically significant (*p*-value = 0.15) (Fig. 4). No correlation was observed between DRC values of the same sample analyzed under different blood storage conditions.

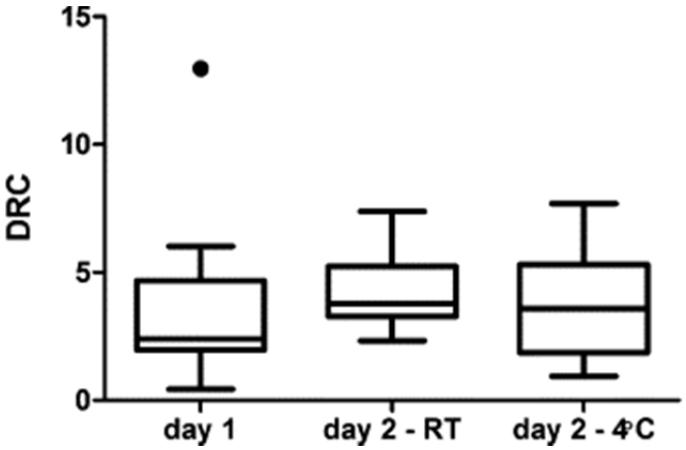

Fig. 4.

Effect of different blood storage condition on DRC measured by aphidicolin-blocked NER comet assay on 10 healthy subjects.

Aphidicolin-block NER comet assay was not affected either by blood storage at room temperature for 24 h or by the storage at 4 °C. However, statistical analysis showed a lack of correlation between DRC values of "day 1" *versus* "day 2-4 °C" (r = 0.694, p-value = 0.06) or "day 2-RT" samples (r = 0.448, p-value = 0.26). Therefore, the performance of NER comet assay on PBMCs separated and cryopreserved soon after blood collection is highly recommended. Anderson et al. analyzed the effect of storage condition (room temperature, 4 °C or −20 °C) on blood to be used in comet assay after exposure to bleomycin or ethylnitrosurea [25]. There was no loss of cell viability at 4 °C or room temperature up to 8 days and minor or no changes in tail moment data of untreated and treated cultures up to 4 days. Nevertheless, data obtained at 4 °C appeared to be more homogeneous, probably because at 4 °C cellular processes might be less active. Moreover, in the case of radiation-induced chromosomal aberration evaluation in human lymphocytes, it was observed that blood storage at temperature of 4 °C, compared to 20 °C, protected the irradiated cells from apoptosis allowing accurate estimation of the real yield of damage [26]. In fact it was previously observed that lymphocytes undergo apoptosis during storage and this loss of viability is accelerated by increasing both temperature and storage time [8].

4. Conclusions

In conclusion, the blood storage conditions are an important factor that may affect the outcome of DRC assays based on the use of PBMCs. In the case of DSBR measurement by H2AX phosphorylation assay, results obtained using either PBMCs from fresh blood or from blood stored for 24 h at room temperature were not significantly different, while storage at 4 °C was not suitable. By contrast, in the case of NER measurement by comet assay, fresh blood results were correlated with those from blood stored for 24 h at

4 °C, while blood storage at room temperature was not suitable. Finally, in the case of OGG activity assay and mRNA expression analysis the storage of blood samples for 24 h (particularly at 4 °C) increased the variability of the measurements. The use of PBMCs from fresh blood is therefore the recommended practice for these assays.

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