Archives of Toxicology (2021) 95:1843–1851
https://doi.org/10.1007/s00204-021-03009-z

PROTOCOLS

Applicability of flow cytometry γH2AX assay in population studies: suitability of fresh and frozen whole blood samples

Blanca Laffon[1,2] · **María Sánchez-Flores**[1,2,3,4] · **Natalia Fernández-Bertólez**[1,2] · **Eduardo Pásaro**[1,2] · **Vanessa Valdiglesias**[2,5]

Received: 27 October 2020 / Accepted: 16 February 2021 / Published online: 23 February 2021

Abstract

Phosphorylation of H2AX histone (γH2AX) represents an early event in the DNA damage response against double-strand breaks (DSB); hence, its measurement provides a surrogate biomarker of DSB. Recently, we reported initial steps in the standardization of γH2AX assay in peripheral blood leukocytes (PBL), addressing the possibility of using cryopreserved samples, and the need of phytohaemagglutinin (PHA) stimulation prior analysis (Toxicol Sci 2015, 144:406-13). Validating the use of whole blood samples as cell specimen for this assay would be particularly useful for human population studies. Hence, in the current study we determined for the first time the feasibility of whole blood samples, both fresh and frozen, to be used in the γH2AX assay, evaluated by flow cytometry, and the convenience of PHA stimulation. Freshly collected and cryopreserved whole blood samples were treated with bleomycin (BLM), actinomycin-D (Act-D) and mitomycin C (MMC); half of the samples were previously incubated with PHA. Results were compared with those from PBL. Negative responses in MMC treatments were probably due to the quiescence of unstimulated cells, or to the short treatment time in PHA stimulated cells. Fresh whole blood samples exhibited a more intense response to BLM and Act-D treatments in stimulated cells, probably due to DSB indirectly produced from other less relevant types of DNA damage. Results obtained in frozen whole blood samples indicate that PHA stimulation is not advisable. In conclusion, this study demonstrates that whole blood samples can be used to assess DSB-related genotoxicity by the flow cytometry γH2AX assay.

Keywords γH2AX assay · DNA damage response · Genomic instability · Genotoxicity · Population studies · Whole blood samples

Introduction

H2AX is a histone variant ubiquitously distributed and expressed throughout the genome. In mammals, 75–98% of H2A is represented by H2A1 and H2A2, whereas 2–25% of all nucleosomes contain H2AX, depending on cell type (Mischo et al. 2005). H2AX takes part in the DNA damage response machinery of mammalian cells and facilitates the repair of clastogenic DNA double-strand breaks (DSB) (Bonner et al. 2008). When a DSB is produced in the DNA, the carboxyl-tail of H2AX is rapidly phosphorylated at Serine 139, to become γH2AX (Rogakou et al. 1998). The phosphorylation of H2AX occurs over a large region of chromatin surrounding the DSB, over a megabase, and can be visualized by the formation of a nuclear foci (Rogakou and Sekeri-Pataryas 1999). This process plays a key role in the recruitment to the site of damage of DNA repair proteins and signalling factors that are required downstream in

✉ Blanca Laffon
blaffon@udc.es

1 Grupo DICOMOSA, Centro de Investigaciones Científicas Avanzadas (CICA), Departamento de Psicología, Facultad de Ciencias de La Educación, Universidade da Coruña, Campus Elviña s/n, 15071 A Coruña, Spain

2 Instituto de Investigación Biomédica de A Coruña (INIBIC), AE CICA-INIBIC. Oza, 15071 A Coruña, Spain

3 Environmental Health Department, National Health Institute, Rua Alexandre Herculano 321, 4000-055 Porto, Portugal

4 EPIUnit—Instituto de Saúde Pública, Universidade Do Porto, Rua das Taipas, n◦ 135, 4050-600 Porto, Portugal

5 Grupo DICOMOSA, Centro de Investigaciones Científicas Avanzadas (CICA), Departamento de Biología, Facultad de Ciencias, Universidade da Coruña, Campus A Zapateira s/n, 15071 A Coruña, Spain

the DNA damage response, in some cases through physical interactions with the proteins (Paull et al. 2000; Stucki et al. 2005). Therefore, phosphorylation of H2AX represents an early event in the DNA damage response (DDR) against DSB, and its measurement provides a surrogate biomarker of DSB with the potential to identify genotoxic exposures. Indeed, comparisons of γH2AX assay with several DNA damage biomarkers—including Ames test, mouse lymphoma assay, chromosome aberration assay, micronucleus test, and comet assay—showed that analysis of γH2AX by flow cytometry is able to detect both pro-genotoxic chemicals (those that require metabolic activation) and genotoxic agents (Zhou et al. 2006; Watters et al. 2009; Smart et al. 2011). Although no formal guidelines have been approved for the γH2AX assay for regular genotoxicity studies, this biomarker has been suggested as a new standard genotoxicity assay (Khoury et al. 2020).

Since the role of γH2AX was first elucidated around two decades ago, an immunocytochemical assay with antibodies recognizing γH2AX emerged as the gold standard for in situ detection of DNA damage, specifically DSB. More recently, a flow cytometry assay was developed for the assessment of γH2AX (Tanaka et al. 2009; Watters et al. 2009). The use of flow cytometry for the evaluation of H2AX phosphorylation allows the assay to be readily standardised. Flow cytometry provides an automated platform that is reproducible, practical, investigator independent, and may take into account variations due to cell-cycle effects. Thus, H2AX phosphorylation can be measured in situ, in chromatin of individual cells, with high sensitivity and accuracy, and the expression of γH2AX can be directly correlated, within the same cells, with their DNA content, induction of apoptosis, or any other cell attribute of interest. Besides, statistical power and, consequently, results robustness, are highly increased since a much larger number of data can be collected (usually 10,000 events in flow cytometry as opposed to 50 nuclei in the microscope scoring). Besides, γH2AX loci counting and flow cytometry analysis showed a high correlation, suggesting that γH2AX scoring by flow cytometry has the potential for high-throughput analysis (Watters et al. 2009; Nikolova et al. 2014).

Recently, we reported initial steps in the standardization of γH2AX assay protocol in peripheral blood leukocytes (PBL), to be employed as a rapid screening tool for genotoxicity or genomic instability in human population studies. Some of the most critical issues limiting the use of this approach were addressed, including the possibility of using cryopreserved samples, and the need of phytohaemagglutinin (PHA) stimulation prior analysis, to force cells to leave quiescence and enter the cell cycle (Sánchez-Flores et al. 2015). Using fresh or cryopreserved PBL samples for γH2AX analysis in human biomonitoring studies depends on the feasibility of processing samples immediately after their collection. Besides, the choice on PBL stimulation with PHA was recommended to be made during the study design, according to the mechanism of production of DSB expected in the particular study population. However, the process of isolating PBL from whole blood samples takes some time and can be limiting in huge population studies when large numbers of samples are collected the same day and must be processed in a short time. Moreover, PBL isolation requires a quite large volume of blood, also restricting the number of biomarkers that can be determined in the blood sample collected. Therefore, validating the use of whole blood samples as cell specimen for the γH2AX assay, not previously addressed, would be particularly useful for human biomonitoring studies.

On this basis, the main objective of this study was to determine for the first time the feasibility of whole blood samples, both fresh and frozen, to be used as cell biomatrices in the yH2AX assay, as evaluated by flow cytometry, also testing the convenience of PHA stimulation of the cells prior to the assay. To this aim, freshly collected and cryopreserved whole blood samples were treated with three well-known genotoxic chemicals: bleomycin (BLM), actinomycin-D (Act-D) and mitomycin C (MMC). Samples were used either directly after collection/thawing or after a 24 h period in cell culture medium in the presence of PHA to allow cell cycle stimulation (Fig. 1). Results were compared with those obtained from PBL, the biomatrix commonly used in the γH2AX assay in human biomonitoring studies.

Material and methods

Chemicals

BLM (CAS number 11056-06-7), Act-D (CAS number 50-76-0), MMC (CAS number50-07-7), RNase A, and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (Madrid, Spain). RPMI 1640, heat-inactivated foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, and PHA were obtained from Gibco (Thermo Fisher Scientific, Madrid, Spain). Stock solutions of BLM, MMC and Act-D were prepared in sterile distilled water (dH_2O) prior their use.

Sample collection and processing

Peripheral blood samples were collected from three healthy non-smoker female volunteers (27–44 years old) by venipuncture into Vacutainer tubes containing heparin as anticoagulant, and into BD Vacutainer® CPT™ with sodium heparin (Becton Dickinson). The study followed ethical criteria established by the Helsinki declaration. Written consent was obtained from each donor prior to joining the study. Whole

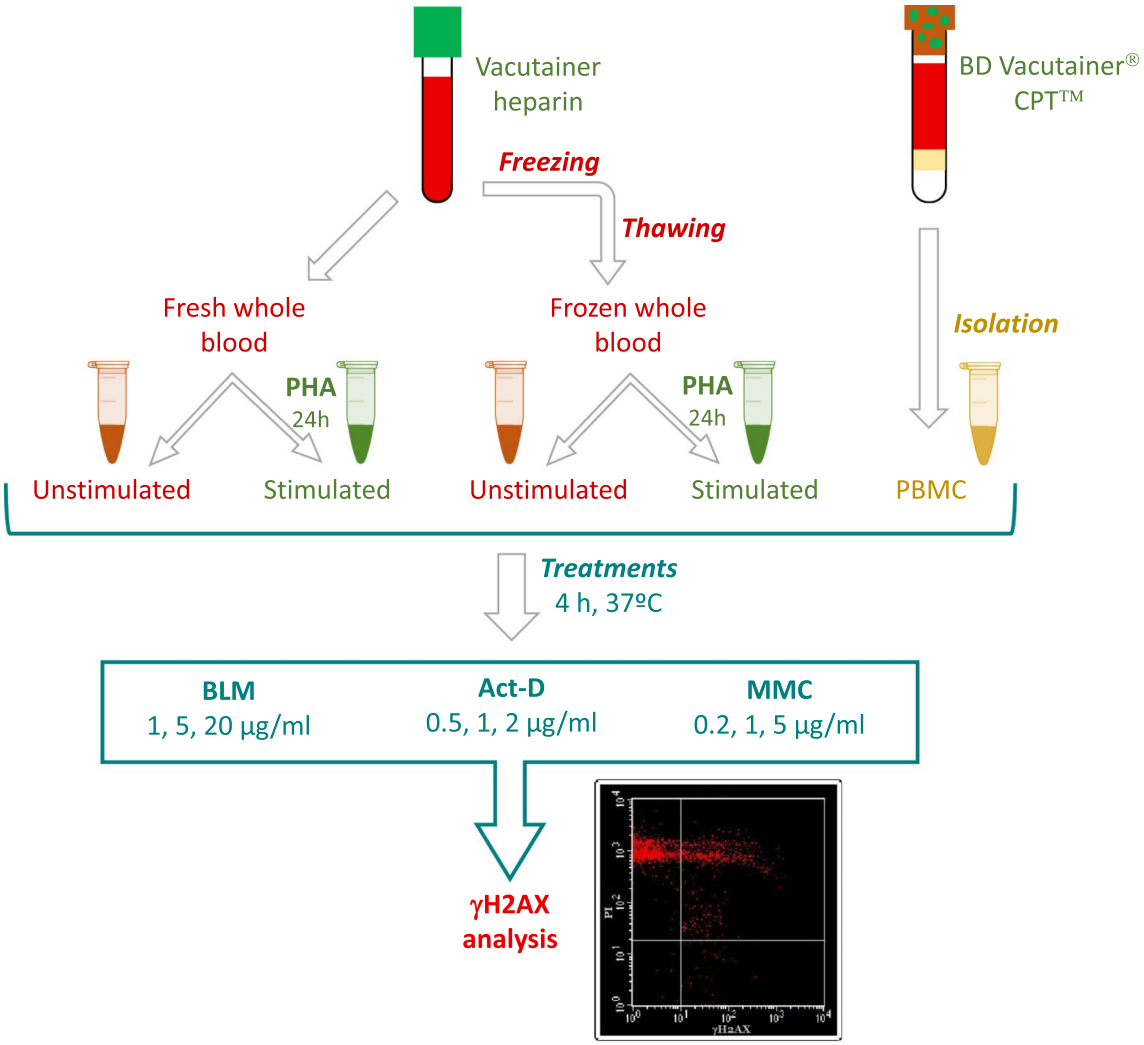

Fig. 1 Scheme of the experimental design of the study. *PHA* phytohaemagglutinin, *PBL* peripheral blood leucocytes, *BLM* bleomycin, *Act-D* actinomycin D, *MMC* mitomycin C

blood samples from Vacutainer tubes were divided in two fractions: one fraction was used in fresh, and the other one was aliquoted and frozen 50:50 in a cryopreserving solution (DMSO/RPMI 1640, 20:80) at − 80 ºC for at least one month. PBL (lymphocytes and monocytes) were isolated from BD Vacutainer® CPT™ tubes according to the manufacturer's instructions.

Treatments

Fresh PBL (2.5×10^5 cells in 200 µl RPMI 1640), fresh blood (100 µl + 100 µl RPMI 1640) and frozen blood (200 µl, quickly thawed at 37 ºC) were resuspended in 800 µl of RPMI 1640 medium containing 15% FBS, 1% L-glutamine (200 mM), and 1% penicillin (5000 U/ml)/streptomycin (5000 µg/ml), in the presence or absence of 1% PHA, depending on whether they would be stimulated or not. Stimulated samples (fresh and frozen whole blood) were incubated prior treatments for 24 h at 37 ºC in the presence of PHA.

Biological samples were treated with three different concentrations of each genotoxic agent (1% of the final volume in medium with or without PHA, depending on each particular case) for 4 h at 37 ºC: BLM (1, 5, 20 µg/ml), Act-D (0.5, 1, 2 µg/ml), and MMC (0.2, 1, 5 µg/ml); dH_2O was employed as a negative control. Chemicals, treatment doses and exposure times used were selected on the basis of previous studies (Valdiglesias et al. 2011; Sánchez-Flores et al. 2015). Cytotoxicity was below 20% in all cases, as confirmed by Trypan blue exclusion technique.

γH2AX analysis

The γH2AX analysis was performed following the protocol described by Tanaka et al. (2009) and Watters et al. (2009), with certain modifications (Sánchez-Flores et al. 2015). In brief, after the treatments, cell suspensions were centrifuged and the supernatant was removed. An additional step was added to lyse erythrocytes in the fresh blood samples by treating them with BD Pharm lyse™ (BD Bioscience) for 8 min in the dark. Cell pellets were fixed in 1% *p*-formaldehyde. Subsequently, cells were centrifuged and the remaining cell pellets were postfixed with cold 70% ethanol (− 20 ºC) and stored at 4ºC overnight. Then cell suspensions were centrifuged, washed in phosphate-buffered solution (PBS), and incubated with 100 µl anti-human γH2AX-Alexa Fluor 488-conjugated antibody (Becton Dickinson) (1:20 dilution in 1% bovine serum albumin [BSA] in PBS). After a new centrifugation step, cells were suspended in PBS containing 0.1 mg/ml RNase A and 40 µg/ml PI. The flow cytometry analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson). The lymphocyte population was gated according to complexity (side scattering) and size (forward scattering). At least 10,000 events in the lymphocyte region were acquired, obtaining data from FL1 (γH2AX-Alexa Fluor 488) and FL2 (PI) detectors. The software Cell Quest Pro (Becton Dickinson) was used to analyse the data; the percentage of gated cells positive for both γH2AX and PI with regard to the total lymphocytes gated was calculated.

Statistical analysis

Each experiment was performed in duplicate, and three independent experiments were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error. Differences among groups were tested with the Kruskal–Wallis test, and two by two were evaluated by Mann–Whitney *U*-test. Associations between two variables were analysed by Spearman's correlation. Statistical significance was considered at $P < 0.05$. Statistical analyses were performed using the IBM SPSS software package V. 21.

Results

In this study, the suitability of whole blood samples to be used in the γH2AX assay, as evaluated by flow cytometry was evaluated. As a reference, results were compared to those obtained from isolated PBL, a well-established biomatrix for this assay (Sánchez-Flores et al. 2015).

Figure 2 shows data obtained in the samples treated with BLM. Progressive and significant increases in %γH2AX with regard to the control were observed in PBL exposed to this chemical, and the dose–response relationship was also significant ($r = 0.897$, $p < 0.01$). In unstimulated fresh blood samples, increases in histone phosphorylation were detected at the two highest BLM concentrations, although significance was reached only at the highest dose; concentration-effect association was significant ($r = 0.651$, $p < 0.01$). Responses of stimulated fresh blood and both frozen blood samples to BLM treatment were more intense, displaying significantly higher values at all concentrations. Significant dose–response relationships were obtained in the two former cases ($r = 0.797$, $p < 0.01$ for stimulated fresh blood samples, and $r = 0.716$, $p < 0.01$ for unstimulated frozen blood samples).

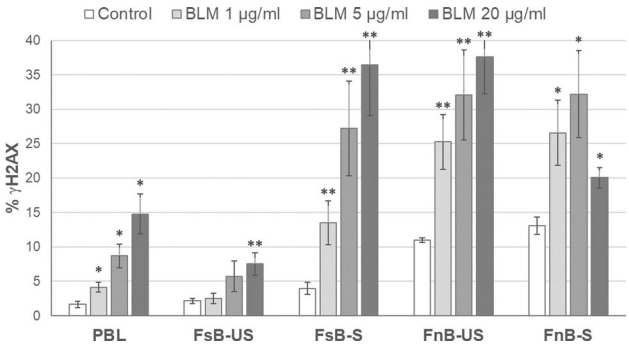

Fig. 2 Results of γH2AX assay in the different sample types treated with bleomycin. Bars represent mean standard error. $*P < 0.05$; $**P < 0.01$, significant difference with regard to the corresponding control. *BLM* bleomycin, *PBL* peripheral blood leucocytes, *FsB-US* unstimulated fresh whole blood, *FsB-S* stimulated fresh whole blood, *FnB-US* unstimulated frozen whole blood, *FnB-S* stimulated frozen whole blood

Results obtained in samples exposed to Act-D are gathered in Fig. 3. Significant increases in histone phosphorylation as compared to the control were obtained in PBL at all Act-D concentrations, and dose–effect relationship was evident ($r = 0.812$, $p < 0.01$). Higher values of %γH2AX were also observed in unstimulated and stimulated fresh blood samples and in unstimulated frozen blood samples treated with Act-D, significant in all cases excepting for the lowest Act-D dose in unstimulated fresh blood. Significant concentration–response effects were found ($r = 0.509$, $p < 0.05$ for unstimulated fresh blood, $r = 0.630$, $p < 0.01$ for stimulated fresh blood, and $r = 0.727$, $p < 0.01$ for unstimulated frozen blood). Still, the intensity of response was notably lower in the former sample type. In contrast, stimulated frozen blood samples responded mildly to Act-D treatment, showing slight increases in %γH2AX with regard to the control, mostly not significant.

Exposure of the different sample types to MMC produced the results represented in Fig. 4. Although in general, and similarly to the responses observed in BLM and Act-D

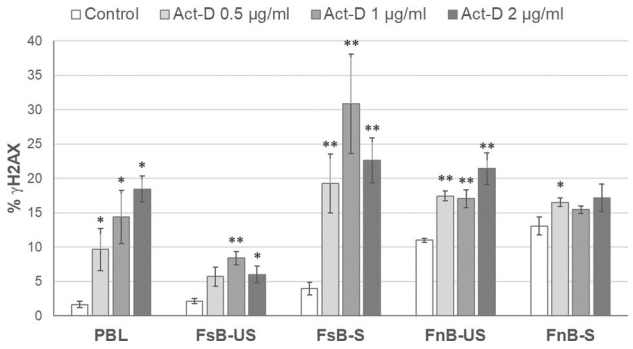

Fig. 3 Results of γH2AX assay in the different sample types treated with actinomycin D. Bars represent mean standard error. $*P<0.05$; $**P<0.01$, significant difference with regard to the corresponding control. *Act-D* actinomycin D, *PBL* peripheral blood leucocytes, *FsB-US* unstimulated fresh whole blood, *FsB-S* stimulated fresh whole blood, *FnB-US* unstimulated frozen whole blood, *FnB-S* stimulated frozen whole blood

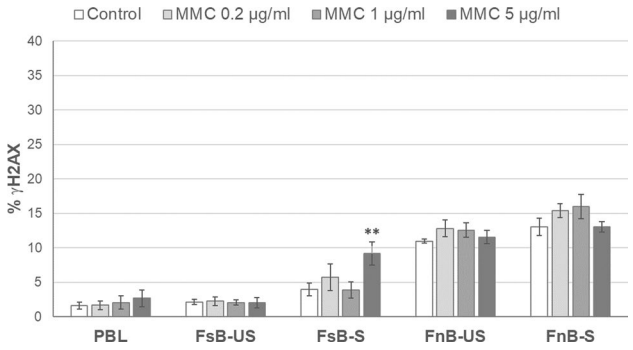

Fig. 4 Results of γH2AX assay in the different sample types treated with mitomycin C. Bars represent mean standard error. $**P<0.01$, significant difference with regard to the corresponding control. *MMC* mitomycin C, *PBL* peripheral blood leucocytes, *FsB-US* unstimulated fresh whole blood, *FsB-S* stimulated fresh whole blood, *FnB-US* unstimulated frozen whole blood, *FnB-S* stimulated frozen whole blood

treatments, the intensity of the response of PBL and unstimulated fresh blood samples is markedly lower than in the other sample types, no increases in histone phosphorylation were obtained with regard to the controls when cells were treated with MMC at any dose, excepting for the highest dose in stimulated fresh blood.

Figure 5 depicts the comparison of the background levels of H2AX phosphorylation in all the negative controls employed (PBL, unstimulated and stimulated fresh blood, and unstimulated and stimulated frozen blood). Similar values were observed in PBL and unstimulated fresh blood samples. Level of %γH2AX in stimulated fresh blood samples was slightly higher than these two, but not significantly different. Oppositely, both unstimulated and stimulated frozen blood samples presented histone phosphorylation

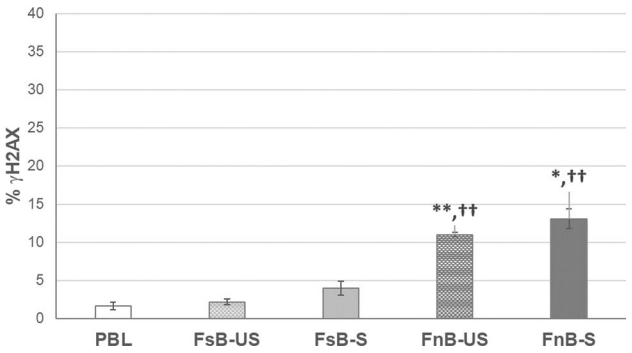

Fig. 5 Comparison between the basal γH2AX levels in all sample types tested (negative controls). Bars represent mean standard error. $*P<0.05$, $**P<0.01$, a significant difference with regard to peripheral blood leucocytes (PBL); $^{\dagger\dagger}P<0.01$, significant difference with regard to the corresponding fresh sample. *FsB-US* unstimulated fresh whole blood; *FsB-S* stimulated fresh whole blood, *FnB-US* unstimulated frozen whole blood, *FnB-S* stimulated frozen whole blood. No significant differences were found in the comparisons between stimulated and unstimulated samples (within fresh or frozen whole blood samples)

values significantly higher than PBL and the corresponding fresh samples. No differences were observed between unstimulated and stimulated samples of the same type (fresh or frozen).

Discussion

DSB are the least frequent but most harmful type of DNA damage since inaccurate DSB repair can lead to genome rearrangements and even a single unrepaired DSB could result in cell death (Yamamoto et al. 2011). There is consensus that γH2AX represents an early response of cells to genotoxic stress that results directly from DSB and/or blocked replication forks that generate single-stranded DNA and, indirectly, upon replication fork collapse, generates DSB (Nikolova et al. 2014). γH2AX assay is one of the most sensitive and specific assays for detecting DSB; it allows the detection of a single DSB since H2AX molecules are phosphorylated in a wide area around the damaged site (Rothkamm and Löbrich 2003). This resolution is impossible to achieve with the conventional assays, including the neutral comet assay. Hence, it is an ideal assay for assessing biologically relevant and severe genotoxic activity in human population studies. Indeed, this technique has a promising potential for an extensive use in environmental and occupational surveillance, and also in the diagnosis, management and prevention of a variety of diseases. Thus, it has been used as a biomarker of genomic instability in cancer development and progression, in evaluating cancer patient sensitivity and responsiveness to ionizing radiation and chemotherapy, and in other non-neoplastic chronic diseases,

especially those related to premature aging and DNA repair defects (reviewed in Valdiglesias et al. 2013a). Nevertheless, the need to isolate PBL from blood samples to conduct the assay limits its applicability in epidemiological studies in terms of time and blood sample size; the possibility of directly using whole blood for the assay would greatly facilitate its use in both clinical and experimental settings. Therefore, in this study, we aimed at determining the feasibility of whole blood samples, both fresh and frozen, for flow cytometry γH2AX assay, as well as valuing the need of stimulating the cells with PHA prior analysis.

The three genotoxic agents used were chosen on the basis of their different mechanisms for induction of DNA DSB, directly or indirectly. BLM is a well-known clastogenic chemical; it behaves as a radiomimetic agent capable of directly inducing a wide spectrum of mutagenic lesions—including DNA base damage, abasic sites, and alkali-labile sites—which eventually result in DNA single-strand breaks (SSB) and DSB (Milić and Kopjar 2004; Chen and Stubbe 2005). Consequently, BLM is commonly used as DNA damage inducer and positive control in genotoxicity assays (Laffon et al. 2010; Valdiglesias et al. 2013b). BLM-induced clastogenicity is replication-independent (Povirk and Austin 1991). The five different sample types tested in this study displayed a progressive response of H2AX phosphorylation to increasing BLM concentrations, with unstimulated fresh whole blood cells showing the lowest sensitivity, likely related to the fact that progress through the cell cycle gives the opportunity to slighter types of DNA damage to be converted into DSB. Positive γH2AX responses in the flow cytometry and the immunocytochemical assays to BLM treatment, at doses similar to the ones used in this study, were previously reported in unstimulated human peripheral lymphocytes (Scarpato et al. 2011), C18-4 spermatogonial cells (Liu et al. 2014), and L5178Y mouse lymphoma cells (Watters et al. 2009).

Act-D is a cross-linking agent used for the treatment of a number of different cancer types such as sarcomas, paediatric solid tumours (*e.g.* Wilms' tumour), testicular cancer, and choriocarcinoma. It intercalates into DNA strands, preferentially into GC rich sequences, preventing the unwinding of the double helix and interfering with RNA polymerase progression, thereby inhibiting transcription (Sobell 1985; Bensaude 2011). Besides, Act-D targets topoisomerases (Kathiravan et al. 2013), enzymes that facilitate DNA unwinding—necessary for transcription and replication processes—by relieving the topological tension in the DNA and removing supercoiling. Act-D extends the half-life of the covalent topoisomerase-DNA complex, stabilizing the nicked intermediates produced and preventing the relegation step (Trask and Muller 1988; Wassermann et al. 1990), thus leading to SSB and DSB (Ross and Bradley 1981; Tewey et al. 1984). PBL, both fresh whole blood samples, and unstimulated frozen whole blood cells responded positively to Act-D; among them, the lowest sensitivity and the poorer dose–response coefficient corresponded again to unstimulated fresh whole blood cells. Stimulated frozen whole blood cells barely reacted to Act-D treatment. Other studies had previously described the induction of H2AX phosphorylation by Act-D treatment at doses ranging from 0.05 to 10 μg/ml in HeLa cells (Mischo et al. 2005) or in resting PBL (Porcedda et al. 2006), by immunocytochemical analysis of γH2AX foci.

MMC is an antitumour antibiotic drug that has been used to treat different cancers including breast, bladder, oesophagus, stomach, pancreas, lung, anal and liver cancers. MMC is extensively used as clastogenic agent and micronuclei inducer in many in vitro and in vivo studies (Valdiglesias et al. 2011; Kataria et al. 2016), and is recommended as a positive control chemical in OECD Test Guidelines for mammalian cell genotoxicity tests (Guidelines Nos. 473, 474, 475, 476, 483, 487, 490). MMC acts as an alkylating agent capable of covalently binding DNA and inducing inter- and intra-strand crosslinks (Roh et al. 2008), although the former are predominant (Avendaño and Menéndez 2015). Treatment with alkylating agents or crosslinkers requires cell cycle progression and transit through S phase for DSB formation and γH2AX expression (Banáth et al. 2010), since DSB arise when a SSB, crosslinked DNA, or damaged DNA base meet a replication fork (Limoli et al. 2002). Indeed, Al-Minawi et al. (2009) reported that MMC induced DSB are the result of the collapse of inter-strand crosslink-stalled replication forks, and showed that MMC induced DSB are prevented by the arrest of replication elongation. PBL and cells from unstimulated whole blood samples are quiescent cells, and thus DSB are not expect to be produced by MMC treatment; consequently, negative responses were observed in all MMC concentrations tested. Stimulated whole blood cells entered the cell cycle after 24 h in the presence of PHA. Since following this incubation treatment with MMC lasted only for 4 h, it is likely that this time was not long enough for replication to be advanced, and thus DSB could not be generated from damaged bases and crosslinks. Only cells from stimulated fresh whole blood samples exposed to the highest MMC concentration could exhibit a significant although a slight increase in H2AX phosphorylation. Therefore, negative phosphorylation responses observed in this study when cells were treated with MMC were probably due to the quiescence of unstimulated cells, or to the short time elapsed from treatment initiation to analysis in PHA stimulated cells. In primary mouse embryonic fibroblasts treated with 3 μM MMC (1 μg/ml), the peak time for γH2AX foci was reported to be around 12 h (Niedernhofer et al. 2004), being the doubling time for these cells 45 h, nearly two-fold the lymphocyte doubling time (Hu et al. 2012). And agreeing with the current results, A172 glioblastoma cells (doubling

time approximately 40 h) treated for 3 h with 0.2–35 µg/ml MMC did not show any significant increase in %γH2AX positive cells (Laffon et al. 2017).

Comparison of the basal levels of H2AX phosphorylation in PBL with the whole blood samples indicates that fresh whole blood samples, both unstimulated and stimulated, are not different from PBL, and their γH2AX background is low. Both control frozen whole blood samples show a significant rise in the %γH2AX positive cells, probably due to extra DNA damage induced during the freezing and thawing processes. Nevertheless, their phosphorylation levels are still low enough to allow detecting additional increases related to any DSB-producing genotoxic activity. In our previous study we reported that, when using cryopreserved lymphocytes for this assay, PHA stimulation is required since unstimulated cells show too high basal damage (Sánchez-Flores et al. 2015). Current results demonstrate that this is not the case of cryopreserved whole blood samples; hence, this is an important time-saving advantage of using this type of sample instead of cryopreserved lymphocytes, together with avoiding the lymphocyte isolation procedure.

As a whole, fresh whole blood samples exhibited a more intense response to BLM and Act-D treatments when cells had been stimulated with PHA. Although both quiescent and cycling cells (in all cell cycle phases, including mitosis) have shown generation of γH2AX in response to DNA damage (Giunta and Jackson 2011), the levels of phosphorylation have been reported to be higher in PHA stimulated lymphocytes than in unstimulated cells when exposed, for instance, to camptothecin (Tian et al. 2011) or to X-rays (Hamasaki et al. 2007; Tian et al. 2011). This is probably related to the fact that in cycling cells DSB are also indirectly produced from other less relevant types of DNA damage. Therefore, if the purpose of a study is to assess just directly produced DSB, no PHA stimulation should be used with fresh whole blood samples. Stimulating cells from this sample type would allow additional detection of other DNA lesions (e.g. damaged bases, cross-links, SSB…) that will become DSB during cell cycle progression. Therefore, the decision on stimulating fresh whole blood samples will depend on the type of damage to be detected. Results obtained in frozen whole blood samples were less uniform when cells were stimulated with PHA (absence of significant dose–response relationship in all cases). Hence, PHA stimulation is not recommended when the γH2AX assay is carried out with frozen whole blood samples.

In conclusion, this study demonstrates that whole blood samples can be used to assess genotoxicity related to the production of DSB by the flow cytometry γH2AX assay. Besides, both stimulated fresh and unstimulated frozen whole blood cells displayed a more marked response to BLM and Act-D treatments than PBL, thus increasing the sensitivity for detecting early repair response to DSB. Further investigations in epidemiological studies will contribute to standardize the use of whole blood as a suitable sample type in the γH2AX assay and to define its utility in different experimental and clinical settings.

Funding This work was supported by Xunta de Galicia [ED431B 2019/02], Ministerio de Educación, Cultura y Deporte [BEAGAL18/00142 to V.V], and Deputación Provincial de A Coruña [to M.S.-F. and N.F.-B.].

Data availability Not applicable.

Code availability Not applicable.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval The manuscript does not contain clinical studies or patient data.

Consent to participate All volunteers gave their informed consent prior to their inclusion in the study.

Consent for publication Not applicable.

References

Al-Minawi AZ, Lee YF, Håkansson D, Johansson F, Lundin C, Saleh-Gohari N, Schultz N, Jenssen D, Bryant HE, Meuth M, Hinz JM, Helleday T (2009) The ERCC1/XPF endonuclease is required for completion of homologous recombination at DNA replication forks stalled by inter-strand cross-links. Nucleic Acids Res 37:6400–6413. https://doi.org/10.1093/nar/gkp705

Avendaño C, Menéndez JC (2015) Anticancer drugs that interact with the DNA minor groove. In: Medicinal chemistry of anticancer drugs. Elsevier, pp 243–271. https://doi.org/https://doi.org/10.1016/B978-0-444-62649-3.00006-5

Banáth JP, Klokov D, MacPhail SH, Banuelos CA, Olive PL (2010) Residual γH2AX foci as an indication of lethal DNA lesions. BMC Cancer 10:4. https://doi.org/10.1186/1471-2407-10-4

Bensaude O (2011) Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity? Transcription 2:103–108. https://doi.org/10.4161/trns.2.3.16172

Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y (2008) γH2AX and cancer. Nat Rev Cancer 8:957–967. https://doi.org/10.1038/nrc2523

Chen J, Stubbe J (2005) Bleomycins: towards better therapeutics. Nat Rev Cancer 5:102–112. https://doi.org/10.1038/nrc1547

Giunta S, Jackson SP (2011) Give me a break, but not in mitosis: the mitotic DNA damage response marks DNA double strand breaks with early signaling events. Cell Cycle 10:1215–1221. https://doi.org/10.4161/cc.10.8.15334

Hamasaki K, Imai K, Nakachi K, Takahashi N, Kodama Y, Kusunoki Y (2007) Short-term culture and γH2AX flow cytometry determine differences in individual radiosensitivity in human peripheral T lymphocytes. Environ Mol Mutagen 48:38–47. https://doi.org/10.1002/em.20273

Hu J, Hu S, Ma Q, Wang X, Zhou Z, Zhang W, Sun X, Zhu W, Qian H, Xu W (2012) Immortalized mouse fetal liver stromal cells support growth and maintenance of human embryonic stem cells. Oncol Rep 28:1385–1391. https://doi.org/10.3892/or.2012.1909

Kataria SK, Chhillar AK, Kumar A, Tomar M, Malik V (2016) Cytogenetic and hematological alterations induced by acute oral exposure of imidacloprid in female mice. Drug Chem Toxicol 39:59–65. https://doi.org/10.3109/01480545.2015.1026972

Kathiravan MK, Khilare MM, Nikoomanesh K, Chothe AS, Jain KS (2013) Topoisomerase as target for antibacterial and anticancer drug discovery. J Enzyme Inhib Med Chem 28:419–435. https://doi.org/10.3109/14756366.2012.658785

Khoury L, Zalko D, Audebert M (2020) Evaluation of the genotoxic potential of apoptosis inducers with the γH2AX assay in human cells. Mutat Res - Genet Toxicol Environ Mutagen 852:1–10. https://doi.org/10.1016/j.mrgentox.2020.503165

Laffon B, Fernández-Bertólez N, Costa C, Pásaro E, Valdiglesias V (2017) Comparative study of human neuronal and glial cell sensitivity for in vitro neurogenotoxicity testing. Food Chem Toxicol 102:120–128. https://doi.org/10.1016/j.fct.2017.02.005

Laffon B, Valdiglesias V, Pásaro E, Méndez J (2010) The organic selenium compound selenomethionine modulates bleomycin-induced DNA damage and repair in human leukocytes. Biol Trace Elem Res 133:12–19. https://doi.org/10.1007/s12011-009-8407-9

Limoli CL, Giedzinski E, Bonner WM, Cleaver JE (2002) UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA doublestrand breaks, γ-H2AX formation, and Mre11 relocalization. Proc Natl Acad Sci USA 99:233–238. https://doi.org/10.1073/pnas.231611798

Liu M, Hales BF, Robaire B (2014) Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. Biol Reprod 90:1–10. https://doi.org/10.1095/biolreprod.114.117754

Milić M, Kopjar N (2004) Evaluation of in vitro genotoxic activity of bleomycin and mitomycin C in human lymphocytes using the alkaline comet assay. In: Arhiv za Higijenu Rada i Toksikologiju. pp 249–259

Mischo HE, Hemmerich P, Grosse F, Zhang S (2005) Actinomycin D induces histone γ-H2AX foci and complex formation of γ-H2AX with Ku70 and nuclear DNA helicase II. J Biol Chem 280:9586–9594. https://doi.org/10.1074/jbc.M411444200

Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF, de Wit J, Jaspers NGJ, Beverloo HB, Hoeijmakers JHJ, Kanaar R (2004) The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. Mol Cell Biol 24:5776–5787. https://doi.org/10.1128/mcb.24.13.5776-5787.2004

Nikolova T, Dvorak M, Jung F, Adam I, Krämer E, Gerhold-Ay A, Kaina B (2014) The γH2AX assay for genotoxic and nongenotoxic agents: comparison of H2AX phosphorylation with cell death response. Toxicol Sci 140:103–117. https://doi.org/10.1093/toxsci/kfu066

Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 10:886–895. https://doi.org/10.1016/S0960-9822(00)00610-2

Porcedda P, Turinetto V, Lantelme E, Fontanella E, Chrzanowska K, Ragona R, De Marchi M, Delia D, Giachino C (2006) Impaired elimination of DNA double-strand break-containing lymphocytes in ataxia telangiectasia and Nijmegen breakage syndrome. DNA Repair (Amst) 5:904–913. https://doi.org/10.1016/j.dnarep.2006.05.002

Povirk LF, Austin FMJ (1991) Genotoxicity of bleomycin. Mutat Res Genet Toxicol 257:127–143. https://doi.org/10.1016/0165-1110(91)90022-N

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 273:5858–5868. https://doi.org/10.1074/jbc.273.10.5858

Rogakou EP, Sekeri-Pataryas KE (1999) Histone variants of H2A and H3 families are regulated during in vitro aging in the same manner as during differentiation. Exp Gerontol 34:741–754. https://doi.org/10.1016/S0531-5565(99)00046-7

Roh DS, Cook AL, Rhee SS, Joshi A, Kowalski R, Dhaliwal DK, Funderburgh JL (2008) DNA cross-linking, double-strand breaks, and apoptosis in corneal endothelial cells after a single exposure to mitomycin C. Investig Opthalmology Vis Sci 49:4837. https://doi.org/https://doi.org/10.1167/iovs.08-2115

Ross WE, Bradley MO (1981) DNA double-strand breaks in mammalian cells after exposure to intercalating agents. BBA Sect Nucleic Acids Protein Synth 654:129–134. https://doi.org/10.1016/0005-2787(81)90145-3

Rothkamm K, Löbrich M (2003) Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci USA 100:5057–5062. https://doi.org/10.1073/pnas.0830918100

Sánchez-Flores M, Pásaro E, Bonassi S, Laffon B, Valdiglesias V (2015) γH2AX assay as DNA damage biomarker for human population studies: defining experimental conditions. Toxicol Sci 144:406–413. https://doi.org/10.1093/toxsci/kfv011

Scarpato R, Verola C, Fabiani B, Bianchi V, Saggese G, Federico G (2011) Nuclear damage in peripheral lymphocytes of obese and overweight Italian children as evaluated by the γ-H2AX focus assay and micronucleus test. FASEB J 25:685–693. https://doi.org/10.1096/fj.10-168427

Smart DJ, Ahmedi KP, Harvey JS, Lynch AM (2011) Genotoxicity screening via the γH2AX by flow assay. Mutat Res—Fundam Mol Mech Mutagen 715:25–31. https://doi.org/10.1016/j.mrfmmm.2011.07.001

Sobell HM (1985) Actinomycin and DNA transcription. Proc Natl Acad Sci USA 82:5328–5331. https://doi.org/10.1073/pnas.82.16.5328

Stucki M, Clapperton JA, Mohammad D, Yaffe MB, Smerdon SJ, Jackson SP (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell 123:1213–1226. https://doi.org/10.1016/j.cell.2005.09.038

Tanaka T, Halicka D, Traganos F, Darzynkiewicz Z (2009) Cytometric analysis of DNA damage: Phosphorylation of histone H2AX as a marker of DNA double-strand breaks (DSBs). In: Methods Mol Biol. pp 161–168. https://doi.org/https://doi.org/10.1007/978-1-59745-190-1_11

Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226:466–468. https://doi.org/10.1126/science.6093249

Tian M, Feng Y, Min J, Gong W, Xiao W, Li X, Tao D, Hu J, Gong J (2011) DNA damage response in resting and proliferating peripheral blood lymphocytes treated by camptothecin or X-ray. J Huazhong Univ Sci Technol—Med Sci 31:147–153. https://doi.org/10.1007/s11596-011-0241-6

Trask DK, Muller MT (1988) Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. Proc Natl Acad Sci USA 85:1417–1421. https://doi.org/10.1073/pnas.85.5.1417

Valdiglesias V, Costa C, Sharma V, Kiliç G, Pásaro E, Teixeira JP, Dhawan A, Laffon B (2013a) Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. Food Chem Toxicol J 57:352–361. https://doi.org/10.1016/j.fct.2013.04.010

Valdiglesias V, Giunta S, Fenech M, Neri M, Bonassi S (2013b) γH2AX as a marker of DNA double strand breaks and genomic

instability in human population studies. Mutat Res Mutat Res 753:24–40. https://doi.org/10.1016/j.mrrev.2013.02.001

Valdiglesias V, Laffon B, Pasaro E, Mendez J (2011) Evaluation of okadaic acid-induced genotoxicity in human cells using the micronucleus test and γH2AX analysis. J Toxicol Environ Heal—Part A Curr Issues 74:980–992. https://doi.org/10.1080/15287394.2011.582026

Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y (1990) Effects of morpholinyl doxorubicins, doxorubicin, and Actinomycin D on mammalian DNA topoisomerases I and II. Mol Pharmacol 38:38–45

Watters GP, Smart DJ, Harvey JS, Austin CA (2009) H2AX phosphorylation as a genotoxicity endpoint. Mutat Res Toxicol Environ Mutagen 679:50–58. https://doi.org/10.1016/j.mrgentox.2009.07.007

Yamamoto KN, Hirota K, Kono K, Takeda S, Sakamuru S, Xia M, Huang R, Austin CP, Witt KL, Tice RR (2011) Characterization of environmental chemicals with potential for DNA damage using isogenic DNA repair-deficient chicken DT40 cell lines. Environ Mol Mutagen 52:547–561. https://doi.org/10.1002/em.20656

Zhou C, Li Z, Diao H, Yu Y, Zhu W, Dai Y, Chen FF, Yang J (2006) DNA damage evaluated by γH2AX foci formation by a selective group of chemical/physical stressors. Mutat Res—Genet Toxicol Environ Mutagen 604:8–18. https://doi.org/10.1016/j.mrgentox.2005.12.004