Video Article

Measuring DNA Damage and Repair in Mouse Splenocytes After Chronic *In Vivo* Exposure to Very Low Doses of Beta- and Gamma-Radiation

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Abstract

Low dose radiation exposure may produce a variety of biological effects that are different in quantity and quality from the effects produced by high radiation doses. Addressing questions related to environmental, occupational and public health safety in a proper and scientifically justified manner heavily relies on the ability to accurately measure the biological effects of low dose pollutants, such as ionizing radiation and chemical substances. DNA damage and repair are the most important early indicators of health risks due to their potential long term consequences, such as cancer. Here we describe a protocol to study the effect of chronic *in vivo* exposure to low doses of γ - and β -radiation on DNA damage and repair in mouse spleen cells. Using a commonly accepted marker of DNA double-strand breaks, phosphorylated histone H2AX called γ H2AX, we demonstrate how it can be used to evaluate not only the levels of DNA damage, but also changes in the DNA repair capacity potentially produced by low dose *in vivo* exposures. Flow cytometry allows fast, accurate and reliable measurement of immunofluorescently labeled γ H2AX in a large number of samples. DNA double-strand break repair can be evaluated by exposing extracted splenocytes to a challenging dose of 2 Gy to produce a sufficient number of DNA breaks to trigger repair and by measuring the induced (1 hr post-irradiation) and residual DNA damage (24 hrs post-irradiation). Residual DNA damage would be indicative of incomplete repair and the risk of long-term genomic instability and cancer. Combined with other assays and end-points that can easily be measured in such *in vivo* studies (e.g., chromosomal aberrations, micronuclei frequencies in bone marrow reticulocytes, gene expression, etc.), this approach allows an accurate and contextual evaluation of the biological effects of low level stressors.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52912/>

Introduction

Significant controversy over the potential harmful effects of either low or very low doses of ionizing radiation and public's fear of radiation, driven by images of the Hiroshima and Nagasaki atomic bombings and of rare nuclear plant accidents (exacerbated by mass media), has led to very strict radiation protection regulation and standards that are potentially not scientifically justified. In the last three decades, numerous reports have documented both the lack of harmful and the presence of potentially beneficial biological effects induced by low dose radiation¹⁻⁴. The major radiation health risk factor is the probability of cancer, estimated based on epidemiological studies of atomic bomb survivors receiving high or medium doses of radiation. Linear extrapolation of these data (so called linear-no-threshold or LNT model) is used to estimate risks of cancer at low doses. However, this approach has not received world-wide scientific acceptance and is heavily debated⁵.

It is evident that more studies are required to clarify this issue and possibly improve radiation protection standards. Such studies should involve chronic treatments (best approximation of environmental and occupational exposures), *in vivo* animal models (best for extrapolating effects to human) and such end-points as DNA damage rates, DNA repair and mutagenesis. It is known that DNA is the primary target for damaging radiation effects and incomplete or mis-repair may lead to mutagenesis and cancer development⁶.

DNA double-strand breaks (DSB) are one of the most deleterious types of DNA lesions and may lead to cell death and tumorigenesis⁷. It is, therefore, important to be able to reliably and accurately measure the level of DSB after exposure to low dose radiation and/or other stressors, such as chemical pollutants. One of the most sensitive and specific markers of DNA DSB is phosphorylated histone H2AX, called γ H2AX⁸, yet other markers and methods have been suggested^{9,10}. It is estimated that thousands of H2AX molecules, in the vicinity of an induced DSB, are involved in the formation of γ H2AX enabling the detection of individual DSB by immunofluorescent labeling with an anti- γ H2AX antibody and fluorescence microscopy¹¹. The response is very quick, reaching its maximum between 30 and 60 min. Evidence exists that γ H2AX facilitates repair of DNA DSB by attracting other repair factors to the sites of breaks and by modifying chromatin structure to anchor broken DNA ends and provide access for other repair proteins (reviewed in¹²). Upon completion of repair of DNA DSB, γ H2AX gets de-phosphorylated and/or undergoes degradation, and newly synthesized H2AX molecules replace γ H2AX in the affected areas of chromatin¹⁰. Monitoring formation and loss of γ H2AX can, therefore, provide an accurate estimate of DNA DSB repair kinetics. This approach has been used to study repair of DSB

in various human tumor cell lines irradiated with high doses of radiation and its rate and residual DSB levels have been shown to correlate with radiosensitivity¹³⁻¹⁵.

We modified this experimental approach and applied it to an *in vivo* mouse study to examine the effects of low doses of chronic γ - and β -irradiation on DNA DSB levels and repair (**Figure 1**). Firstly, we demonstrate a method to perform a long-term chronic exposure of mice to β -radiation either emitted by tritium (hydrogen-3) in the form of tritiated water (HTO) or as organically bound tritium (OBT) dissolved in drinking water. The two forms are expected to accumulate and/or distribute differently in the body and therefore, produce different biological effects. Both forms are potential hazards in nuclear industry. This treatment is paralleled by chronic exposure to γ -radiation at an equivalent dose rate to allow a correct comparison of the two radiation types, which is crucial for the evaluation of their relative biological effectiveness. Beta-radiation is composed of electrons, making it very different from the γ -radiation, high energy photons. Due to this difference, β -radiation represents mostly internal health hazard and may produce different biological effects compared to γ -radiation. This complication resulted in significant controversies over the regulation of exposure to β -radiation emitted by HTO. Thus, regulatory levels of HTO in drinking water for the public vary from 100 Bq/L in Europe to 75,000 Bq/L in Australia. It is, therefore, important to compare biological effects of HTO to equivalent doses of γ -radiation. Secondly, the rate of DNA DSB is measured in isolated splenocytes upon the completion of chronic exposures using immunofluorescently labeled γ H2AX detected by flow cytometry. This allows the evaluation of the extent of DNA damage inflicted by the *in vivo* exposures. However, it is sensible to expect that such low level exposures may not produce any detectable rates of DNA DSB; instead, some hidden changes/responses may be expected that would affect the cells' ability to repair DNA damage. These changes, if found, may be either stimulatory (producing a beneficial effect) or inhibitory (producing a deleterious effect). The proposed protocol allows revealing such changes by challenging the extracted splenocytes with a high dose of radiation that produces a significant amount of damage (e.g., 2 Gy producing approximately 50 DNA DSB per cell or a 3 - 5 fold increase in total γ H2AX level). Subsequently, the formation and loss of γ H2AX, reflecting the initiation and completion of DSB repair, is monitored by flow cytometry. In this way, not only basal and treatment-induced levels of DNA DSB can be measured, but also its potential effect on the cells' ability to respond and repair DNA damage induced by much higher stressor levels.

Protocol

All mouse handling and treatment procedures should adhere to rules set forth by the legislature and/or animal care programs and approved by a local animal care committee. All methods described in this protocol were performed in accordance with the guidelines of the Canadian Council on Animal Care with the approval of the local animal care committee.

CAUTION: All work with radioactivity (including, but not limited to handling of tritium, external γ -irradiation, handling radioactive animal tissues, bedding waste) should adhere to rules set forth by the legislature and/or radiation protection authorities and be performed by authorized personnel in a certified laboratory and/or facility.

1. Animal Work

1. Animal Grouping
 1. If mice are arriving from an external organization, acclimatize animals for at least 10 days in the animal facility. If the study includes multiple treatment groups, ensure that the animal supplier can send all animals as a single batch.
 2. Randomly allocate mice to treatment groups of 10 mice per group. If using male mice of the C57BL/6 strain, have the animal supplier pre-arrange randomization of very young C57BL/6 males in their facility to mitigate aggression issues.
2. Chronic Exposure of Mice to Internal β -Radiation
 1. Prepare working concentrations of tritium (10 kBq/L and 1 MBq/L) in mouse drinking water by diluting the original stock of HTO and OBT (a mixture of tritiated proline, alanine and glycine amino acids) solutions.
 2. Validate the final concentrations of tritium in the drinking water by measuring radioactivity using a liquid scintillation counter. Adjust concentrations, if required.
 3. Treat animals for one month by providing access ad libitum to the tritium water. If treatment water gets low during the two week period, add more water. Otherwise, change bottles at two weeks.
 4. Treat sham-irradiated control mice identically. Keep them in a separate "clean" room or on a separate "clean" rack under positive air pressure to avoid tritium cross-contamination from the air.
3. Chronic Exposure of Mice to External γ -Radiation

Note: γ -irradiation facility/equipment will vary depending on the location.
4. Using available methods of dosimetry, determine the distances from a γ -radiation source that have radiation fields producing the desired dose rates. Ensure homogeneity of the radiation fields within the areas covering the animal cages.

Note: 1.7 μ Gy/h is equivalent to a dose rate produced by 1 MBq/L tritium in the body.
5. Expose mice by placing mouse cages at the distances determined in step 1.3.1 for one month. Minimize daily interruption of γ -irradiation to 30 min. Use thermoluminescence dosimeters to measure total absorbed doses.

2. Sacrifices and Sampling

1. Prepare sterile surgical instruments, 60 mm petri dishes with inserted 70-micron cell strainers, 15 ml and 1.5 ml tubes, RPMI media supplemented with 5% fetal bovine serum (FBS) and place all within a biological safety cabinet. Ensuring sterility of the work is crucial.
2. Dispense 5 ml of RPMI media into each 60 mm petri dish.
3. Sacrifice mice using a method approved by the local animal care committee. Cervical dislocation is a good choice for the splenocyte work described in this protocol. However, if blood samples for other assays (e.g., for the mFISH, the cytochalasin block micronucleus or other common genotoxicity assays) are required, use intra-cardiac puncture after anaesthesia with isoflurane.

4. Place the animal in dorsal recumbency, with the head pointing away from you. Spray the mouse down with 70% ethanol. Using forceps, grab the skin anteriorly to the urethral opening and make a small incision with scissors in the perineal region.
 1. From this incision, cut along the ventral midline to the chest cavity, being careful to only cut the skin and not the muscle wall underneath. Dissect the skin away from the midline.
 2. Grasp the abdominal wall with forceps and cut along the median axis of the muscular wall to open up the abdominal cavity.
 5. Excise the spleen by lightly gripping it with sterile forceps and gently pulling on it while simultaneously cutting away the connective tissue with scissors.
 6. Cut a small piece of the spleen (approximately 1/10 of the spleen) and place in a 1.5 ml tube. Snap freeze in liquid nitrogen for subsequent storage at -80 °C for supplementary assays.
 7. Place the remainder of the spleen into a cell strainer inside a 60 mm petri dish with 5 ml of pre-dispensed RPMI media.
 8. Proceed to the next mouse.
- Note: Extracted spleens can be kept in 60 mm dishes with media (for up to 2 hrs) while the rest of the mice are sacrificed. Depending on the number of people participating, between 5 and 15 mice can be processed in one day.

3. Preparation of Splenocyte Cultures

1. Homogenize the spleen by mincing inside a cell strainer with sterile forceps with a curved end.
2. Remove the cell strainer and collect the filtered cell suspension from the 60 mm dish into a 15 ml tube.
3. Rinse the cell strainer with 5 ml of media to collect the remainder of the cells.
4. Centrifuge tubes at 300 x g for 5 min at RT.
5. Decant supernatant and re-suspend pellet in 10 ml of RPMI.
6. Centrifuge tubes at 300 x g for 5 min at RT.
7. Decant supernatant and re-suspend pellet in 5 ml of RPMI.
8. Transfer 4 ml of cell suspension into a 25 ml tissue culture flask and remove to a CO₂ incubator (37 °C, 5% CO₂, 80% humidity)

4. Challenging Irradiation and Fixing

1. Transfer the remaining 1 ml of cell suspension from step 3.8 into a 1.5 ml tube on ice and centrifuge at 300 x g for 5 min at 4 °C. DNA damage measured in this sample will represent both treatment-induced damage and t = 0 data-point for the construction of a DNA repair curve.
 2. Carefully aspirate the supernatant using a vacuum pump. Gently re-suspend pellet in 1 ml of TBS buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2.7 mM KCl).
 3. Centrifuge tubes at 300 x g for 5 min at 4 °C. Carefully aspirate the supernatant using a vacuum pump. Gently re-suspend pellet in 300 µl of TBS.
 4. While mixing on a vortex at low speed, add 700 µl of -20 °C 100% ethanol. Close the lid and invert a few times to mix.
 5. Store the samples at -20 °C. Splenocytes fixed in ethanol can be stored at -20 °C for at least 12 months without any noticeable loss in the γH2AX signal.
 6. Irradiate the splenocyte cultures from step 3.8 with a challenging γ-radiation dose of 2 Gy at a dose rate ≥ 200 mGy/min using available irradiation device.
- Note: The purpose of this irradiation is to induce DNA DSB to challenge the repair machinery. X-rays can also be used for this purpose.
7. Immediately return the cultures to a CO₂ incubator.
 8. 1 hr after the challenging 2 Gy irradiation, remove the cultures from the incubator to a biological safety cabinet. To collect a representative sample aliquot, gently re-suspend cells using a pipettor and transfer 1 ml of the cell suspension to a 1.5 ml tube on ice. Return the cell cultures to a CO₂ incubator.
 9. Centrifuge tubes at 300 x g for 5 min at 4 °C. Carefully aspirate the supernatant using a vacuum pump. Gently re-suspend pellet in 1 ml of TBS.
 10. Repeat steps 4.3 - 4.5.
 11. 24 hrs after the challenging 2 Gy γ-irradiation, harvest and fix a t = 24 hrs sample as described in step 4.8 – 4.10.

5. Immunofluorescent Labeling with Anti-γH2AX Antibody

Note: Typically, 15 - 30 samples per day can be immunofluorescently labeled and assayed by flow cytometry in a single run. To ensure an accurate comparison between treatment groups, include sample(s) from each group. See also Reference ¹⁶ for further details.

1. Prepare a labeled set of tubes for the number of samples to be processed and place on ice. Use 12 x 75 mm uncapped glass tubes. Sterility is not required for this work. Include one additional historical control sample for labeling with secondary antibody only ("2nd Ab only"). Use this historical control sample in every flow cytometry run within a study.
2. Add 0.5 ml of ice-cold TBS to each tube.
3. Remove fixed splenocytes from -20 °C, vortex for 5 sec and transfer a 0.5 ml aliquot to prepared tubes with TBS. Place the remainder of samples back into -20 °C storage and keep as a backup sample.
4. Centrifuge splenocytes at 300 x g for 5 min at 4 °C. Decant supernatant and gently re-suspend pellet in 1 ml of ice-cold TBS containing 1% FBS.
5. Centrifuge cells at 300 x g for 5 min at 4 °C. Decant the supernatant and gently re-suspend pellet in 1 ml of TST buffer (0.05% Triton X-100, 2% FBS in TBS). Incubate for 20 min on ice.
6. Centrifuge cells at 300 x g for 5 min at 4 °C. Decant the supernatant and gently re-suspend pellet in 200 µl of primary anti-γH2AX antibody diluted 1:150 in TST buffer. Use 200 µl of TST for the "2nd Ab only" control sample.
7. Position tubes on a rotating shaker at an angle of 45 - 60° and shake for 1.5 h at 300 x g at RT.

8. While tubes are incubating, prepare a sufficient volume (200 μ l/sample) of the goat anti-mouse secondary antibody conjugated with Alexa-488 at a 1:200 dilution in TST buffer.
Note: All work involving Alexa-488 conjugated antibody (steps 5.10 to 5.16 below) should be done under dim light conditions and labeled samples should be protected from light by wrapping tubes in aluminum foil.
9. Once the 1.5 hrs incubation is completed, add 1 ml of ice-cold TBS containing 2% FBS to each tube and centrifuge at 300 x g for 5 min at 4 °C. Decant the supernatant and gently re-suspend pellet in 1 ml of TBS containing 2% FBS.
10. Centrifuge at 300 x g for 5 min at 4 °C. Decant supernatant and gently re-suspend pellet in 200 μ l of the secondary antibody solution from step 5.8.
11. Incubate the samples on a shaking platform for 1 hr as in step 5.7.
12. Add 1 ml of ice-cold TBS containing 1% FBS.
13. Centrifuge at 300 x g for 5 min at 4 °C. Decant supernatant and gently re-suspend pellet in 1 ml of ice-cold TBS.
14. Centrifuge at 300 x g for 5 min at 4 °C. Decant the supernatant and gently re-suspend pellet in 0.5 ml of TBS containing 50 μ g/ml propidium iodide.
15. Incubate for 5 min at RT protected from light.
16. Analyze samples on a flow cytometer using instrument-specific protocols¹⁶. Read at least 10,000 cells per sample.

Representative Results

Figure 2 shows examples of flow cytometry graphs expected for splenocytes prepared using the method described here. Cells are first gated based on the <electronic volume-side scatter> scatter plot (**Figure 2A** and **2D**; electronic volume is equivalent to forward scatter). FL3/propidium iodine histograms (**Figure 2B** and **2E**) confirm normal cell cycle distribution. Mean γ H2AX signal calculated using the FL1 channel confirms a > 2-fold increase in DNA DSB in 2-Gy irradiated cells (**Figure 2F**) compared to the untreated control (**Figure 2C**). Next, we examined the sensitivity of the described method of measuring immunofluorescently labeled γ H2AX by flow cytometry in mouse splenocytes. **Figure 3A** shows γ H2AX levels, indicative of DNA DSB, in mouse splenocytes 1 hr after the *ex vivo* exposure to either low (0.1 Gy) or high (2 Gy) doses of γ -radiation relative to the untreated control. It can be seen that 0.1 Gy induced a slight increase in the DSB level and that increase was statistically significant. The strong 3-fold induction was expected and detected after the 2 Gy exposure. To validate the specificity of γ H2AX immunofluorescent labeling as described in the protocol, labeled cells were examined with a fluorescence microscope. The marked foci-like pattern observed indicates that the fluorescence signal originates from the individual DNA DSB within the chromatin (**Figure 3B**).

Representative results of the assessment of DNA DSB rate and their repair in splenocytes of mice exposed for one month to very low concentrations of tritiated water (10 kBq/L) are presented in **Figure 4**. It can be seen (**Figure 4A**) that although the treatment resulted in a 10% increase of the basal γ H2AX level, the change was not statistically significant (N = 5, one-sample Student's *t* test). Furthermore, the measured formation and loss of γ H2AX after the challenging 2 Gy irradiation, representing the kinetics of DNA DSB repair, was not different between the cells from the control and HTO-treated mice (**Figure 4B**).

Figure 1. Experimental diagram to evaluate the effect of chronic *in vivo* irradiation on DNA damage and DNA repair. After *in vivo* treatment of mice with chronic irradiation for a desired period of time, mice are sacrificed and splenocyte cultures are established and exposed to a challenging irradiation. Splenocyte aliquots are collected and fixed at various times after the challenging dose. Levels of γ H2AX are then measured using immunofluorescent labeling and detection by flow cytometry. The plot on the bottom of the Figure is a schematic of expected data. Gamma-H2AX level drastically increases early after the challenging irradiation followed by a decrease back to the control value, if DNA repair is complete. DNA damage Δ represents the damage produced due to the experimental treatment of mice and DNA repair Δ represents the effect of the treatment of mice on DNA DSB repair capacity. [Please click here to view a larger version of this figure.](#)

Figure 2. Representative flow cytometry raw data. (A, D) Scatter plots and gating regions for splenocytes and cell debris and erythrocytes. (B, E) DNA histograms representing cell cycle distributions. (C, F) γ -H2AX fluorescence histogram and mean fluorescence values. Data in sets (A-C) were obtained from a representative untreated control spleen cells, whereas data in sets (D-F) were obtained from cells irradiated with 2 Gy γ -radiation and harvested 1 hr after the irradiation. [Please click here to view a larger version of this figure.](#)

Figure 3. Detection of γ H2AX by flow cytometry is sensitive and specific. (A) Splenocytes freshly isolated from male CBA mice were irradiated with either 0.1 or 2 Gy of γ -radiation, or sham-treated and allowed to develop γ H2AX response for 1 hr. Cells were then fixed, immunofluorescently labeled with anti- γ H2AX antibody and analyzed by flow cytometry. Normalized mean values $\pm$ SD are shown (N = 12). * and *** signify statistical difference with $p < 0.05$ and $p < 0.001$, respectively (one-sample Student's t -test). (B) Representative microphotographs of untreated control (UT) or 2 Gy irradiated splenocytes immunofluorescently labeled with anti- γ H2AX antibody. The foci-like pattern of green fluorescence confirms the specificity of the immunofluorescent labeling for γ H2AX. [Please click here to view a larger version of this figure.](#)

Figure 4. Representative results showing the lack of effect produced by 1-month exposure of mice to tritiated water (HTO) on DNA DSB level and repair. (A) Splenocytes were isolated from mice treated with 10 kBq/L of HTO in drinking water and control animals. The level of γ H2AX was measured using immunofluorescent labeling and flow cytometry as described in the protocol. Mean γ H2AX fluorescence levels relative to those of control $\pm$ SD are shown (N = 5). These data were generated from the $t = 0$ time-point that was used in **Figure 3B** for the evaluation of DNA DSB repair. (B) Isolated splenocytes were exposed to a challenging 2 Gy dose of γ -radiation and aliquots of cells were fixed at times $t = 0, 1$ and 24 hrs after the challenging irradiation. Mean fluorescence of γ H2AX relative to its own $t = 0$ control is shown $\pm$ SD (N = 5). [Please click here to view a larger version of this figure.](#)

Discussion

The protocol presented in this paper is useful for conducting large scale mouse *in vivo* studies examining the genotoxic effects of low levels of various chemical and physical agents, including ionizing radiation. Our unique specific pathogen-free animal facility equipped with a GammaBeam 150 irradiator and a 30 meter long irradiation hall allows conducting life-long studies involving low or very low dose rate irradiations on hundreds or even thousands of mice. Smaller irradiation facilities for chronic low dose irradiation can be set up in a university or hospital environment where radiobiology studies are conducted routinely and radiation protection guidelines/regulations are implemented. However, stricter regulations normally apply to facilities designed for internal animal irradiation where handling of radionuclides, such as tritium, is carried out. Although rare, laboratories certified for such internal radiation exposure studies exist in various research institutions world-wide and their unique capabilities can be used for studies described in this protocol.

Within the protocol, we use the immunofluorescent labeling of γ H2AX to accurately evaluate DNA DSB rates produced by experimental exposures. More importantly, the introduction of a challenging irradiation delivered *ex vivo* to extracted splenocytes makes it possible to evaluate the intactness of the DNA DSB response (γ H2AX level at 1 hr after the challenge) and of DNA DSB repair (γ H2AX level at 24 hr compared to 0 hr). It should be noted that quantification of γ H2AX foci per cell by fluorescence microscopy may provide higher sensitivity compared to flow cytometry. For example, doses as low as 0.001 Gy were reported to produce significant increases in the number of γ H2AX foci per cell. However, the use of flow cytometry-based detection of γ H2AX provides advantages over fluorescence microscopy analysis because it requires less time for measurements, it does not require highly specialized/trained personnel and it avoids subjective decision-making related to γ H2AX foci recognition and counting. These advantages are critical for large scale animal studies. A typical animal experiment with 10 treatment groups and 10 mice per group will result in $100 \times 3 = 300$ spleen samples, if the method suggested (**Figure 1**) is followed. To generate data from this number of samples, approximately 100 hrs will be required using flow cytometry. Alternatively, if done by manual microscopic analyses, the same task would take at least 300 hrs, using a conservative estimate of 40 min of highly demanding microscope time per sample. Although attempts to automate analysis and scoring of γ H2AX foci have been made¹⁷⁻¹⁹, they all have a number of limitations. In particular, the round shape and small size of mouse splenocytes and lymphocytes makes it impractical to resolve all individual γ H2AX foci microscopically. While adherent cells have been successfully scored by automated microscopy systems for γ H2AX foci, no success has been demonstrated for either mouse splenocytes or lymphocytes.

The best way to eliminate day-to-day variability in the measured values of γ H2AX signal by flow cytometry is to include experimental group and control samples in the same run and to calculate relative γ H2AX changes within every run. Additional recommendations include: a) a historical control consisting of untreated and 2 Gy irradiated sample is used in every run; b) one person performs all the antibody labeling procedures; c) a single stock of buffers and a single batch of antibody, both primary and secondary, is used for the entire set of samples within one experiment. It is also vital to perform a flow cytometer quality control test recommended by the instrument manufacturer routinely and prior to each run of samples. This is required to verify the instrument's optical alignment and fluidics system and also helps minimizing day-to-day variation in measured γ H2AX fluorescence between samples to be compared or pooled in one group.

It should be pointed out that the use of a larger number of time-points (e.g., 1, 2, 4, 6 hrs) post challenging irradiation may help analyze and measure short term DNA DSB repair kinetics in greater detail¹⁶. However, overall repair potential and the likeliness of delayed health effects would be represented by the residual damage measured only at 24 hrs and compared to the one observed at the initial time point ($t = 0$)¹³⁻¹⁵ (**Figure 4**).

Since basal γ H2AX levels are known to increase in old animals or senescent cells²⁰, for more accurate interpretation of the results obtained in long-term exposure mouse studies (> 6 months) using the described method, the use of a young untreated group, in addition to an age-matched control, would be advisable. Although some reports indicate that γ H2AX may increase in proliferating cells, which would not be related to DNA DSB, this can easily be controlled by measuring S-phase and G2/M-phase cell fractions using DNA histograms (**Figure 2A, D**). If certain experimental conditions still prevent a researcher from using γ H2AX as a marker of DNA damage (due to the reasons outlined above or their combination), another protein forming DNA damage foci, such as 53BP1, can be used²¹.

Although we have not attempted using this method for non-lymphoid tissues, such as muscle, heart, brain and others, it would seem difficult to process them for flow cytometry. Tissue sections and microscopy for γ H2AX foci counting would be the method of choice, if analysis of such tissues is required. However, other lymphoid cells, such as peripheral blood lymphocytes, bone marrow lymphocytes or thymocytes, can be used for measuring γ H2AX levels by the described method. The advantage of using spleen lymphocytes is the relative simplicity of cell suspension preparation and the large number of cells that can be isolated from a spleen. This becomes even more advantageous if additional end-points are included in the study. In our experience, a typical yield of splenocytes per mouse allows for the collection of at least 10 aliquots, each suitable for any of the following assays: flow cytometry, RT-qPCR for gene or miRNA expression, western blot, genomic DNA for CpG methylation assay. Thus, additional splenocyte aliquots collected in parallel to those for the γ H2AX end-point may be used to measure the expression of genes involved in DNA damage signaling and repair responses. Such genes as GADD45A and CDKN1A, that are normally transcriptionally up-regulated in response to cytotoxic treatments²², may provide a clue as to whether cells are properly responding to the challenging stress influence. Similarly, protein levels and post-translational modifications involved in stress responses may be assessed. Finally, it is not expected that any of the steps of the extraction procedure, if performed as described here, can induce γ H2AX in splenocytes.

It would be beneficial and highly recommended to sample additional organs and tissues during sacrifices to supplement DNA damage and repair data obtained for splenocytes as per presented protocol with other end-points. For example, we routinely collect bone marrow cells for the *in vivo* bone marrow micronucleus assay, various tissues for senescence-associated β -galactosidase and DNA repair gene expression measurements. The ability to collect other tissues would depend on the available technical support during sacrifices; however, it can significantly help in the proper interpretation of results and the construction of a much more biologically relevant systemic picture of the response to the low level treatment being investigated.

Disclosures

The authors declare that there are no known competing financial interests.

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