



Impact of the redox environment on propagation of radiation bystander effects: The modulating effect of oxidative metabolism and oxygen partial pressure

Géraldine Gonon, Sonia M de Toledo, Venkatachalam Perumal, Jean-Paul Jay-Gerin, Edouard I Azzam

► To cite this version:

Géraldine Gonon, Sonia M de Toledo, Venkatachalam Perumal, Jean-Paul Jay-Gerin, Edouard I Azzam. Impact of the redox environment on propagation of radiation bystander effects: The modulating effect of oxidative metabolism and oxygen partial pressure. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 2022, 883-884, pp.503559. 10.1016/j.mrgentox.2022.503559 . irsn-03889132

HAL Id: irsn-03889132

<https://irsn.hal.science/irsn-03889132>

Submitted on 7 Dec 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

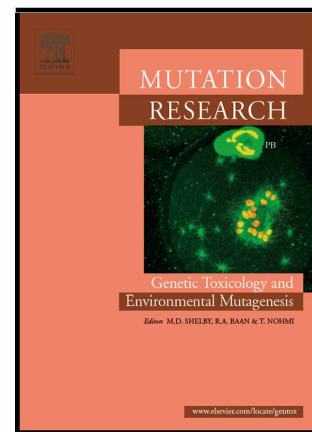
L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Impact of the Redox Environment on Propagation of Radiation Bystander Effects: the Modulating Effect of Oxidative Metabolism and Oxygen Partial Pressure
Redox environment and radiation bystander responses

Géraldine Gonon, Sonia M. de Toledo, Venkatachalam Perumal, Jean-Paul Jay-Gerin, Edouard I. Azzam



PII: S1383-5718(22)00121-8

DOI: <https://doi.org/10.1016/j.mrgentox.2022.503559>

Reference: MUTGEN503559

To appear in: *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*

Received 1 September 2022
date:

Revised date: 9 November 2022

Accepted 11 November 2022
date:

Please cite this article as: Géraldine Gonon, Sonia M. de Toledo, Venkatachalam Perumal, Jean-Paul Jay-Gerin and Edouard I. Azzam, Impact of the Redox Environment on Propagation of Radiation Bystander Effects: the Modulating Effect of Oxidative Metabolism and Oxygen Partial Pressure
Redox environment and radiation bystander responses, *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, (2022)
doi:<https://doi.org/10.1016/j.mrgentox.2022.503559>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Impact of the Redox Environment on Propagation of Radiation Bystander Effects: the Modulating Effect of Oxidative Metabolism and Oxygen Partial Pressure

Géraldine Gonon^{a,b}, Sonia M. de Toledo^b, Venkatachalam Perumal^{c,b}, Jean-Paul Jay-Gerin^d, Edouard I. Azzam^{b,e,2}

^a Institut de Radioprotection et de Sûreté Nucléaire (IRSN), PSE-SANTE/SERAMED/LRAcc, Fontenay-aux-Roses, France

^b Department of Radiology, Rutgers New Jersey Medical School, Newark, NJ, USA

^c Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research (Deemed to be University), Chennai, India

^d Département de médecine nucléaire et de radiobiologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada

^e Radiobiology and Health Branch, Isotopes, Radiobiology & Environment Directorate (IRED), Canadian Nuclear Laboratories (CNL), Chalk River, Ontario, Canada

Running head: Redox environment and radiation bystander responses

Key words: bystander effects, oxygen partial pressure, radiolytic species, dose rate, alpha particles, tritium electrons

^{1,2} Address for correspondence:

Géraldine Gonon
Institut de Radioprotection et de Sûreté
Nucléaire (IRSN)
Laboratoire de radiobiologie des
expositions accidentelles (LRAcc)
B.P.17
92262 Fontenay-aux-Roses, France
Phone: +33 (0)1 58 35 80 93
E-mail: geraldine.gonon@irsn.fr

Edouard I. Azzam

Canadian Nuclear laboratories (CNL)
Radiobiology and Health Branch
Chalk River, ON K0J 1P0
Canada
Phone: 613-584 3311 (ext. 44127)
E-mail: edouard.azzam@cnl.ca;
edouard.azzam@rutgers.edu

Journal Pre-proof

Highlights

- Radiation-induced bystander effects are attenuated at below ambient pO_2 .
- A prooxidant cellular environment amplifies radiation bystander effects.
- Early chemical steps impact the radiation bystander response.
- The bystander effect is independent of radiation dose rate.
- The reported results are relevant to radiation protection and radiotherapy.

ABSTRACT

Redox modulated pathways play important roles in out-of-field effects of ionizing radiation. We investigated how the redox environment impacts the magnitude of propagation of stressful effects from irradiated to bystander cells. Normal human fibroblasts that have incorporated [³H]-thymidine were intimately co-cultured with bystander cells in a strategy that allowed isolation of bystander cells with high purity. The antioxidant glutathione peroxidase (GPX) was maintained either at wild-type conditions or overexpressed in the bystanders. Following 24 h of coculture, levels of stress-responsive p21^{Waf1}, p-Hdm2, and connexin43 proteins were increased in bystander cells expressing wild-type GPX relative to respective controls. These levels were significantly attenuated when GPX was ectopically overexpressed, demonstrating by direct approach the involvement of a regulator of intracellular redox homeostasis. Evidence of participation of pro-oxidant compounds was generated by exposing confluent cell cultures to low fluences of 3.7 MeV α particles in presence or absence of t-butyl hydroperoxide. By 3 h post-exposure to fluences wherein only ~2 % of cells are traversed through the nucleus by a particle track, increases in chromosomal damage were greater than expected in absence of the drug ($p < 0.001$) and further enhanced in its presence ($p < 0.05$). While maintenance and irradiation of cell cultures at low oxygen pressure (pO₂ 3.8 mm Hg) to mimic in vivo still supported the participation of bystander cells in responses assessed by chromosomal damage and stress-responsive protein levels ($p < 0.001$), the effects were attenuated compared to ambient pO₂ (155 mm Hg) ($p < 0.05$). Together, the results show that bystander effects are attenuated at below ambient pO₂ and when metabolic oxidative stress is reduced but increased when the basal redox environment tilts towards oxidizing

conditions. They are consistent with bystander effects being independent of radiation dose rate.

(282/300 words)

Journal Pre-proof

INTRODUCTION

Ionizing radiation-induced bystander effects refer to the emergence of biological changes in non-irradiated cells that are in the vicinity of other cells targeted by radiation (Little 2003). Both harmful and protective bystander effects were described. The type of radiation to which cells are exposed, the size of the absorbed dose, the time at which a particular endpoint is measured after exposure, are factors that impact the nature and magnitude of the induced effect (reviewed in (Buonanno, Gonon et al. 2022)). Harmful bystander effects have been extensively observed when monolayer cultures of various lineages of cells are exposed to low fluences of high-linear energy transfer (LET) particulate radiations such as α particles, wherein only a small fraction of the cells are traversed through the nucleus by a particle track (e.g., (Nagasawa and Little 1992; Azzam, de Toledo et al. 1998; Zhou, Randers-Pehrson et al. 2000; Shao, Furusawa et al. 2003; Gonon, Groetz et al. 2013)). With relevance to clinical exposures, toxic effects were also detected in non-irradiated cells co-cultured with cells exposed to moderate/high doses of various types of radiation, including sparsely ionizing X and γ rays (de Toledo, Buonanno et al. 2017). Notably, bystander effects were detected in tissue models and in partial body-irradiated animals (reviewed in (Buonanno, Gonon et al. 2022)). Together, the findings have highlighted avenues for exploiting the signaling pathways underlying out-of-field effects both for beneficial purposes and providing a mechanistic explanation for the emergence of adverse outcomes following radiotherapeutic treatments or occupational and environmental exposures to densely ionizing radiations (reviewed in (Prise, Folkard et al. 2003; Mothersill and Seymour 2004; Hei, Zhou et al. 2011; Buonanno, Gonon et al. 2022)).

At the onset of efforts to elucidate the mechanisms underlying the radiation-induced bystander phenomenon, it was shown that bystander cells neighboring irradiated cells experienced perturbations in oxidative metabolism that mirrored those detected in the cells traversed by radiation (Lehnert, Goodwin et al. 1997; Narayanan, Goodwin et al. 1997; Azzam, De Toledo et al. 2002; Azzam, de Toledo et al. 2003). This was confirmed by various approaches, among which treatment of cell cultures with antioxidants and inhibition of oxidases. The disproportionate increase in the fraction of cells harboring DNA damage in cultures exposed to low fluences of α particles was significantly reduced when the cultures were pre-incubated with superoxide dismutase (SOD), catalase or when the superoxide generator NAD(P)H oxidase was inhibited. This work highlighted the involvement of reactive oxygen species (ROS), specifically superoxide anion and hydrogen peroxide, in mediating the spread of oxidative stress from irradiated to bystander cells (Narayanan, Goodwin et al. 1997; Azzam, De Toledo et al. 2002).

Partial oxygen pressure (pO_2) in cells is an essential controller of organ function (Carreau, El Hafny-Rahbi et al. 2011). Acute or chronic deviation from homeostatic pO_2 environments results in pathological conditions (Michiels 2004) and influences the response to exogenous challenges whether being environmental or therapeutic (Gray, Conger et al. 1953; Prabhakar and Semenza 2015). Whereas early radiobiological research found that oxygen is a dose modifying factor (Petry 1923), cell cultures for laboratory experiments are still mainly maintained in “classic” incubators under ambient atmospheric oxygen level ($\sim 20.95\%$ or $pO_2 \sim 159$ mm Hg that is often supplemented with 5% CO_2 for a final $pO_2 \sim 141$ mm Hg or 18.6%) (Table 1) (Wenger, Kurtcuoglu et al. 2015; Place, Domann et al. 2017; Ast and Mootha 2019). In vivo, the pO_2 in tissues never

reaches ambient conditions (i.e. 141 mm Hg) (Table 1). For example, the pO_2 of arterial blood is 90-110 mm Hg and of venous blood is ~25-40 mm Hg. In normal tissues, the pO_2 varies between 6 and 34 mm Hg (1-5 % O_2) (Bambrick, Kostov et al. 2011) or even lower for the large intestine (3-11 mm Hg) (Ast and Mootha 2019), a range that often reflects the distance over which oxygen is delivered and consumed. Thus, classical in vitro method of culturing cells derived from human tissue imposes an artificial "hyperoxia" with consequences to the cellular radiation responses, which have not been fully characterized for numerous biological endpoints. Although cells can adapt their physiological functions to ambient pO_2 differently according to their type, the activity of their oxygen and redox-sensitive signaling molecules could be altered (Keeley and Mann 2019). Such variations in the activity of signaling molecules (e.g. transcription factors, enzymes) affects, among other functions, the magnitude and nature of the induced DNA damage as well as the extent of its repair, cell cycle progression, and the intercellular communications impacting the tissue response (Jagannathan, Cuddapah et al. 2016). Consequently, the resulting signaling lesions affected by variations in pO_2 in cells traversed by a radiation track(s) could modulate the nature and concentration of signaling molecules that induce changes in bystander cells. Conversely, the pO_2 of affected bystander cells may greatly modulate their response to the stress communicated from the irradiated cells and their interaction with irradiated and other cells in their vicinity.

While acutely hypoxic cells are more resistant to the effects of sparsely ionizing radiation than their normoxic counterparts (Russo, Mitchell et al. 1985; Brown and Giaccia 1994), a controversy exists as to whether an increase or a decrease in the metabolic production of ROS is involved in hypoxic signaling (Bunn and Poyton 1996;

Chandel, Maltepe et al. 1998; Chandel and Schumacker 2000; Spitz, Azzam et al. 2004). According to Clanton (Clanton 2007), ROS are more likely to be produced in hypoxia when there is both a high reductive capacity (e.g., high NADH/NAD⁺ levels) and sufficient availability of molecular oxygen for reactions linked to the respiratory chain (Chandel, McClintock et al. 2000; Clanton 2007).

It is well-established that exposure of cells to low LET radiation under decreased pO₂ results in reduced cytotoxicity, mutagenicity and DNA breaks (Tisljar-Lentulis, Henneberg et al. 1983; Held, Bren et al. 1986; Grdina, Nagy et al. 1989; Fuciarelli, Wegher et al. 1990; Prise, Folkard et al. 1990; Frankenberg-Schwager, Frankenberg et al. 1991; Denault and Liber 1993; Jagannathan, Cuddapah et al. 2016), which constitutes a major cause of failure in treating tumours with low LET radiation (Sorensen and Horsman 2020). In contrast, resistance of hypoxic mammalian cells to the killing effects of radiation was not detected following exposure to densely ionizing radiations such as α particles (Barendsen, Koot et al. 1966). Interestingly, consistent with previous suggestions (Alper and Bryant 1974; Baverstock and Burns 1981), Monte Carlo simulations of water radiolysis by high LET particulate radiations (4.8 MeV $^4\text{He}^{2+}$ ions (LET ~ 94 keV/ μm), 24 MeV $^{12}\text{C}^{6+}$ ions (LET ~ 490 keV/ μm)) revealed that molecular oxygen is generated around the particle track (Meesungnoen and Jay-Gerin 2009; Zakaria, Colangelo et al. 2020). Whereas in their 2009 study, Meesungnoen and Jay-Gerin showed that the early generation of O₂ occurs around (isolated) individual particle tracks, which is in support of “oxygen-in-the track” concept since dose-rate effects were not considered, in the study of Zakaria, Colangelo et al., the effects of dose rate were considered. In other words, the latter study showed that the early generation of O₂ now extends over the entire irradiated

volume. Regardless, this yield of oxygen provides a suitable explanation for the enhanced toxicity of high LET radiations. Further, under hypoxic condition, the rate of cell growth (Bedford and Mitchell 1974), expression of specific genes (Wilson and Sutherland 1989; Graeber, Peterson et al. 1994; Semenza 2000; Nakayama and Kataoka 2019), ion channels' activity, and DNA repair may be modulated (Lopez-Barneo, Lopez-Lopez et al. 1988; Shimoda and Polak 2011; Kaplan and Glazer 2020), which further contributes to the nature and extent of the cellular radiation response.

The different effects of hypoxia (i.e. pO_2 lower than ambient) on the expression of bystander responses examined in cell cultures exposed to low fluences of high LET radiation are unclear and are beginning to be examined (Zhang, Zhang et al. 2021). The characterization of the bystander effect under conditions that mimic in vivo pO_2 conditions is important for understanding the magnitude of in vitro bystander effects and the possible implications for radiation protection and radiation therapy, in particular when organs that vary in their oxygenation levels are targeted by radiation. Standard protocols for cell culture at ambient pO_2 may or may not accurately simulate in vivo physiological responses to ionizing radiation.

We hypothesize that varying the oxygen pressure at which cells are grown and irradiated results in variable effects on the magnitude of stressful effects propagated to bystander cells. To test this hypothesis, confluent normal human cell cultures maintained in 2-dimensional architecture (2-D) were incubated at 37 °C in a 5 % CO_2 and ~0.5 or ~1 % oxygen (Table 1), in humidified air atmosphere for 48 h before and during exposure in the range of 0.1-10 cGy of 3.7 MeV α particles. At different incubation times in the same atmosphere after exposure, the cells were processed for analyses of biological

endpoints. In the following, the term ‘normoxia’ corresponds to the commonly used oxygen pressure in cell culture incubators maintained at 37 °C in humidified atmosphere at oxygen pressure of 141 mm Hg (i.e., 18.6 % in air or 182 μ M O₂ dissolved in culture medium; Table 1) (Ast and Mootha 2019), whereas ‘hypoxia’ refers to pO₂ lower than ambient (in this study ~0.5 or ~1 % O₂ i.e., ~3.3 mm Hg, ~4.3 μ M or 6.7 mm Hg, 8.7 μ M, respectively; Table 1).

In additional efforts to understand the role of the inherent redox status of cells in propagation of bystander effects, we ectopically over-expressed the antioxidant GLUTATHIONE PEROXIDASE (GPX) gene in cells destined to be bystanders. The latter is an intracellular enzyme that reduces hydrogen peroxide produced during mitochondrial respiration and other enzymatic sources to water (Spitz, Azzam et al. 2004). We also pretreated normal human cell cultures with the oxidizing agent tert-butyl hydroperoxide (t-BOOH) prior to exposure at 37 °C to low fluences of α particles in a 5 % CO₂ in ambient humidified air atmosphere. Whereas glutathione peroxidase catalyzes the reduction of hydroperoxides, incubation of cells with t-BOOH leads to generation of alkoxyl and peroxy radicals in the presence of metal ions. This results in generation of excess ROS, including H₂O₂ (Woodbine, Brunton et al. 2011), which accelerates lipid peroxidation, DNA damage, and causes depletion of glutathione (GSH) (Sandstrom 1991; Guidarelli, Cattabeni et al. 1997; Kim, Kang et al. 1998).

MATERIALS AND METHODS

Cell culture; growth adaptation in low partial oxygen pressure atmosphere

To examine the effects of partial oxygen pressure, a custom-made 'Xvivo' chamber that integrates the latest advances in oxygen monitoring was constructed by BioSpherix Ltd. (Parish, NY) for our specific use (Schematic I, panel A). The chamber is equipped with dissolved oxygen sensors that do not consume oxygen, and with controllers for humidity, carbon dioxide and temperature. Oxygen can be controlled at normobaric pressure to within 0.1 % of any single set point over the entire 0.1 – 99.9 % range. Long-term culturing conditions that permit cells to progressively adapt to various partial oxygen pressures thus can be achieved. To reduce stress associated with transfer of cell cultures from ambient pO_2 to a more hypoxic environment, the pO_2 was decreased in a controlled manner over a 24 h period (Schematic 1, panel C).

Apparently normal AG1522 human diploid skin fibroblasts were obtained from the Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Low passage (p10) confluent cells were used in experiments. For α particle-irradiation, the cells were seeded in specially designed dishes integrating a 1.5 μ m-thick polyethylene terephthalate (PET) growth surface, and fed as described (Gonon, Groetz et al. 2013). After the last feeding, cell cultures were transferred to the controlled atmosphere chamber and placed in a humidified incubator located within the chamber and maintained at 37 °C in the same atmosphere of 5 % CO_2 in humidified ambient air, or to a different incubator within the same chamber where the oxygen concentration was regulated from incubator ambient (141 mm Hg) to ~1 or ~0.5 % in air (6.7 or 3.3 mm Hg) over 24 h (the small volume of 2 mL of culture medium in the circular dishes integrating a 36-mm-diameter

growth surface facilitated its de-oxygenation). Once the desired oxygen level was reached, the cells were further incubated at that atmosphere for additional 24 h. The cultures were exposed to low fluence α particles delivered by an irradiator located within the chamber (Schematic 1, panel C) and remained in the chamber until harvested for analysis of biological endpoints. Because the maximum range of the δ rays produced by 3.7 MeV α particles is only $\sim 0.1 \mu\text{m}$ (Hamm, Turner et al. 1985; Muroya, Plante et al. 2006), it is safe to consider that bystander cells do not receive any dose.

Co-Culture cell system

For experiments involving co-culture of irradiated cells with bystander cells, we used a $10 \mu\text{m}$ -thick Transwell insert with $1\text{-}\mu\text{m}$ pores allowing intercellular communication between the co-cultured cells as we have described (de Toledo, Buonanno et al. 2017). Briefly, AG1522 cells destined to be bystanders were seeded at the bottom side of inverted inserts placed in 6-well plates. The attachment occurred within ~ 40 min. The inserts were then re-inverted and growth medium was added to the 6-well plates. Meanwhile, cells that were grown in flasks in medium containing 37 kBq/mL [^3H]-thymidine were rinsed several times with growth medium (although by the third rinse, no radioactivity was detectable in the rinsing medium, additional four rinses were performed). The labeled or unlabeled and sham-manipulated cells were then seeded, respectively, at confluent density on the top side of the insert with confluent bystander cells growing at the bottom side (Domogauer, de Toledo et al. 2016). Within 2 h after plating, radio-labeled cells adhered and formed functional junctional channels with bystander cells, as was assessed by the transfer of Calcein AM, a green fluorescent gap-

junction permeable dye (Domogauer, de Toledo et al. 2016). Irradiated and bystander cells may also communicate with each other through secreted factors (soluble molecules, exosomes and other extracellular vesicles) transferred across the pores of the membrane. The radio-labeled and bystander cells were left in co-culture for a total of 6 h. Sham-irradiated cells that were handled similarly as the [^3H]-thymidine labelled cells served as control. As the nuclei of the bystander cells growing on the bottom side of the Transwell insert are separated from the irradiated cells by a distance of 10 μm , the ratio of the dose to the labelled and bystander cells is expected to be over 100,000. Therefore, according to Goddu et al., the dose absorbed by the unlabeled bystander cells is expected to be negligible (Goddu, Howell et al. 1994).

Alpha particle irradiation and dosimetry

Exposure to α particles was from a 0.2 mCi ^{241}Am -collimated source at a mean absorbed dose rate of 2 cGy/min as described previously (Gonon, Groetz et al. 2013). Irradiation occurred from below, through the 1.5 μm -thick PET base on which the cells were growing. At the cell layer, the α particles had an estimated average energy of 3.7 MeV with LET calculated to be $\sim 109 \text{ keV}/\mu\text{m}$. Sham-irradiated cell cultures served as control and were handled in parallel with test cultures. Beam uniformity was assessed with CR-39 plastic obtained from Track Analysis Systems, Ltd. (Bristol, UK). Exposure times of 4 s that are commensurate with typical irradiation times used in bystander effect experiments were used to test uniformity of the beam. The plastic was etched according to the manufacturer's instructions. CR-39 plastic is sensitive to α particles and is not sensitive to X rays and γ rays. Thus, only the α particles emitted by ^{241}Am were detected.

The pits created by the etching process were scored at 40 locations (each consisting of 125,000 μm^2) along the entire diameter of the CR-39 plastic corresponding to the diameter of the PET growing surface as we have described (Neti, de Toledo et al. 2004). The α particle fluence was calculated by integrating the counts from 0.2 to 6 MeV during a 200-s acquisition and dividing by the active area of a Canberra (Meriden, CT) CAM300 passive implanted planar silicon (PIPS) detector. The fractions of cells whose nucleus was traversed by a primary particle were calculated according to Charlton and Sephton (Charlton and Sephton 1991) and are described in Table 2.

Western blot analysis

Immunoblotting was performed in protein lysates of whole cells harvested 3 h after irradiation as described previously (Gonon, Groetz et al. 2013). The levels of proteins were quantified with anti-p21^{Waf1} (no. 05-345, Millipore), anti-p-TP53ser15 (no. 9284S, Cell Signaling), anti-p-ERK1/2 (no. 9101S, Cell Signaling), anti-Hdm2 (no. M4308, Sigma) and anti-Connexin43 (GJA1) (no.c6219, Sigma) antibodies. Respective secondary antibodies conjugated with horseradish peroxidase were used to detect the various proteins by chemiluminescence. To verify equal loading of samples, we used over the course of the project either staining of the nitrocellulose membranes with Ponceau S (Sigma) (Romero-Calvo, Ocon et al. 2010), a house-keeping protein (β -tubulin), or a non-specific band that appeared on the autoradiogram following reaction with the secondary antibody as loading control. Experiments were repeated at least 3 times, and representative data are shown in results. Treated samples were compared with the control.

Immunostaining of 53BP1

Following a 3 h incubation after irradiation, cell cultures were fixed in 3.2 % (vol/vol) paraformaldehyde for 10 min at room temperature. Permeabilization, reaction with anti-53BP1 antibody (no. A300-272A; Bethyl) and detection with FITC-conjugated secondary antibody (no. A11037; Invitrogen) were performed as described previously (Gonon, Groetz et al. 2013).

The fractions of cells with 53BP1 foci were evaluated using a UV microscope (Leica DM IL) as previously described (Gonon, Groetz et al. 2013). Each graph is representative of at least three separate experiments. For each experiment, 2 irradiated and 2 control dishes were analyzed. For each dish, more than 3000 cells were scored by eye in more than 40 different fields. Nuclei with atypical size or morphology or those with very high foci counts (presumably appearing in S-phase cells) were not scored (Wilson, Nham et al. 2010). Poisson statistics was used to calculate the standard error associated with the percentage of cells with foci over the total number of cells scored. The Pearson's χ^2 test was used to compare treatment groups versus respective control. A value of $p \leq 0.05$ between groups was considered significant.

Micronucleus Formation

Radiation-induced DNA damage was assessed by measuring the frequency of micronucleus formation by the cytokinesis-block technique (Fenech and Morley 1985). Briefly, 2×10^4 cells were seeded in chamber flaskettes (Nalge Nunc International) in presence of 2 $\mu\text{g/ml}$ cytochalasin B (Sigma) and allowed to progress in the cell cycle. At this concentration, cytochalasin B was not toxic to the cells. After 72 h incubation, the

cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 (1 µg/ml in PBS), and viewed with a fluorescence microscope. At least 1000 binucleated cells per treatment in each experiment were examined. The fraction of binucleated cells in the population was ~40 % in all treatments and only the number of micronuclei per binucleated cell was considered for analysis. Each graph is representative of at least 3 separate experiments, and Poisson statistics was used to calculate the standard error associated with the percentage of micronucleated cells in the total number of binucleated cells scored. Comparisons between treatment groups and respective controls were performed using the Pearson's χ^2 -test. A value of $p \leq 0.05$ between groups was considered significant.

Chemicals: t-butyl hydroperoxide treatment and [³H]-thymidine

t-Butyl hydroperoxide (Sigma/Aldrich, Cat. No. 458139) was dissolved in growth medium that was conditioned by AG1522 cells for 48 h and added to the cell cultures, 1 h before irradiation, at a concentration of 0.5 µM. The cells were incubated in the presence of the drug until they were harvested or fixed 3 h later.

[³H]-thymidine was obtained from Perkin-Elmer Life Sciences (Billerica, MA) as described previously (Gerashchenko and Howell 2005). Cells were treated with [³H]-thymidine at concentration of 5.8 kBq/ml (1.9 µM) as described (Gerashchenko and Howell 2004).

Vectors, Cell Transduction and Measurement of Enzyme Activity

Replication-defective recombinant adenovirus type 5 with the E1 region substituted with the human gene encoding GLUTATHIONE PEROXIDASE (AdGPX)

was obtained from ViraQuest (North Liberty, IA). The infectious units of the adenovirus were typically at 1×10^{10} PFU/ml. GLUTATHIONE PEROXIDASE construct in adenovirus was added to cell cultures in serum free fresh medium for 24 h; the cells were then fed with fresh medium, and experiments were initiated 24 h later as previously described (Autsavapromporn, de Toledo et al. 2011).

To measure glutathione peroxidase activity, AG1522 cell cultures were rinsed with ice-cold PBS and harvested by scraping at 4°C. Pelleted cells ($\sim 10^7$ cells) were rinsed in PBS, re-pelleted and suspended in 50 mM potassium phosphate buffer containing 1.34 mM diethylenetriaminepenta-acetic acid (pH 7.8), and homogenized at 4°C. Protein concentration was determined by the method of Lowry (Lowry, Rosebrough et al. 1951) and total glutathione peroxidase activity was measured by the spectrophotometric method of Lawrence and Burk (Lawrence and Burk 1976) using cumene hydroperoxide as the substrate. One unit of enzymatic activity, expressed as mU/mg protein, is defined as the amount of protein that oxidizes 1 mMol of NADPH/min. Typically, GPX activity was increased by about threefold in AG1522 cells transduced with AdGPX (15 mU/mg in cells transduced with empty vector vs. 45 mU/mg in cells transduced with AdGPX) (de Toledo, Asaad et al. 2006).

RESULTS

Effect of the redox environment on the propagation to bystander cells of stressful effects emanating from irradiated cells

- a- Upregulation of antioxidant defense in bystander cells attenuates the expression of stressful effects propagated from irradiated cells

To investigate the contribution of the redox environment to the propagation of stressful effects from irradiated to bystander cells, we used a genetic approach whereby the antioxidant GLUTATHIONE PEROXIDASE (GPX) gene was ectopically over-expressed in cells destined to be bystanders. This enzyme, present in the cytoplasm and in the mitochondrial matrix (Limon-Pacheco and Gonsebatt 2009), is a primary line of defense that removes peroxides generated as a result of oxidative metabolism by coupling their reduction with the oxidation of glutathione. A Transwell co-culture system whereby bystander cells are in intimate communication with irradiated cells via gap junctions and secreted factors was used (Autsavapromporn, de Toledo et al. 2011; Buonanno, de Toledo et al. 2011) (Figure 1, panel A). Normal confluent AG1522 bystander cells (~98% in G₀/G₁ phase of the cell cycle) were co-cultured for 6 h with AG1522 cells that had previously incorporated [³H]-thymidine (~250 mBq/cell). The cells radiolabeled with tritiated thymidine ($\tau_{1/2}$ = 12.3 years) are still being irradiated when they are co-cultured with bystander cells; however, the very short range (~1 μ m in water) of the low-energy β particles emanating from tritium (average energy ~ 5.68 keV) is restricted to the labeled cells only (Browne, Firestone et al. 1986; Gerashchenko and Howell 2004) and no extracellular cross-irradiation of unlabeled bystander cells is expected to occur (see

Methods section). Nevertheless, due to co-culture with labelled cells, increases in the levels of p21^{waf1}, p-Hdm2, and connexin43 occurred in bystander cells (2 to 3 folds increases) (Figure 1, panel B). When AG1522 bystander cells were transduced with adenovirus construct for GLUTATHIONE PEROXIDASE, a significant decrease in the levels of the latter stress-responsive proteins occurred compared to control non-transduced cells (representative results are shown in Figure 1).

b- Effect of pro-oxidant environment: the modulating effect of t-BOOH

To further understand the role of the redox environment, we increased the cellular level of ROS above normal conditions by incubating confluent monolayer AG1522 cell cultures with the oxidizing agent t-BOOH. Three hours after exposure to low absorbed doses of 3.7 MeV α particles, the cells were harvested and assayed for micronucleus and 53BP1 foci formation. Under normoxia, relative to control, significant increases in micronuclei occurred after exposure to 0.2, 1 or 10 cGy ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) (Figure 2, panel A). The magnitude of the increase in cell cultures exposed to 10 cGy relative to 0.2 cGy was 3.8-fold greater, and in cultures exposed to 10 cGy relative to 1 cGy was 2.4-fold greater. Applying dosimetric calculations, the mean fraction of cells in the population traversed by a particle track through the nucleus at 10 cGy is 50-fold greater than exposure to 0.2 cGy, and 10-fold greater than exposure to 1 cGy (Table 2), which is suggestive of DNA damage occurring in bystander cells as was observed in other studies using broadbeam or microbeam irradiations (Nagasawa and Little 1992; Azzam, de Toledo et al. 1998; Zhou, Suzuki et al. 2001; Azzam, De Toledo et al. 2002; Autsavapornporn, Suzuki et al. 2013). When oxidative stress was initiated by

incubating cell cultures maintained in an atmosphere of 18.6 % O₂ in the presence of non-toxic concentration of 0.5 μ M t-BOOH in the growth medium beginning 1 h before irradiation and until harvesting 3 h later, the increases in micronuclei formation were greater in presence than in absence of the drug, with the differences being significant after exposure to 1 cGy ($p < 0.05$) (Figure 2, panel A).

Another surrogate marker of DNA damage is the p53 binding protein-1 (53BP1) that localizes at the site of DNA breaks and forms discrete foci within minutes after irradiation (Schultz, Chehab et al. 2000; Rappold, Iwabuchi et al. 2001; Asaithamby, Uematsu et al. 2008). In cell cultures maintained and exposed to 0.2 or 1 cGy in an atmosphere of 21 % O₂, where only 1.6 % or 8 % of cell nuclei are traversed by an α particle, respectively, the percentage of cells with 53BP1 foci was increased at 3 h by 1.21 and 1.37 relative to control, ($p < 0.001$ and $p < 0.001$, respectively) (Figure 2, panel B). Incubation and irradiation of the cell cultures in presence of t-BOOH resulted in significant increases in both control and irradiated cultures when compared to maintenance and irradiation of the cultures in absence of the drug ($p < 0.001$) (Figure 2, panel B). Collectively, the results support a significant role of the redox environment in induction of DNA damage in irradiated cells and its propagation to bystander cells.

c- Impact of pO₂

Effect on micronucleus formation

To mimic an aspect of in vivo conditions, we cultured and irradiated AG1522 skin fibroblasts with 3.7 MeV α particles at a pO₂ of 0.5 % O₂, which avoids artificial hyperoxia imposed by classical cell culture. Compared to maintenance and irradiation of the cultures at ambient O₂ level, the increases of micronucleus formation as a function of

absorbed dose (0.2, 1 and 10 cGy) were attenuated, but remained significantly greater than control ($p < 0.05$, $p < 0.001$ and $p < 0.001$ respectively) (Figure 2, panel C). Comparing the effect of 0.5 and 18.6 % oxygen atmosphere, the difference in induction of micronuclei was significant only after irradiation of the cultures with 10 cGy ($p < 0.05$) (Figure 2, panel C). Furthermore, compared to irradiation at ambient pO_2 , 53BP1 foci formation, an indicator of DNA damage, was reduced when the cultures were maintained and irradiated at $\sim 0.5\%$ O_2 atmosphere ($p < 0.001$ at 1 cGy), Figure 2, panel D). Interestingly, the background formation of 53BP1 foci was also reduced.

Effect on stress-responsive protein levels

Normal human fibroblasts sense oxygen levels and respond to hypoxic conditions through the regulation of multiple signaling pathways (Giaccia, Simon et al. 2004). We investigated the modulation of stress-responsive proteins in α particle-exposed confluent AG1522 fibroblast cultures under low (0.5 %) or ambient (18.6 %) oxygen atmosphere. The levels of p-TP53^{ser15}, p21^{Waf1}, and p-ERK1/2 were examined in whole cell lysates by western blot analyses at 3 h after exposure. In the representative results shown in Figure 3, the relative changes in protein levels were compared to non-irradiated samples maintained at 18.6 % O_2 . Consistent with previous data, maintenance and exposure of cell cultures to 0.2, 1 or 10 cGy, under ambient oxygen condition, resulted in increased p-TP53^{ser15}, p21^{Waf1} and p-ERK1/2 levels (Figure 3, panel A). When the cell cultures were maintained under reduced O_2 concentration, the basal level of p-TP53^{ser15} and p21^{Waf1} were lower than in ambient atmosphere. In irradiated samples, the increases in protein levels relative to control were also less than under normoxia. These differences were

particularly noticeable at 10 cGy where ~80 % of the cells were presumably traversed by one or more α particle tracks through the cell nucleus. In case of the transcriptional regulator p-ERK1/2, greater increase occurred under reduced pO_2 than ambient conditions in irradiated samples (a factor of 2 at 0.2 cGy) (Figure 3, panel A) highlighting that enhanced ERK1/2 phosphorylation may be associated with propagation of radiation-induced stressful effect under hypoxia.

Intercellular-communication via gap-junctions was shown to be highly significant in the propagation of stressful effects from irradiated to bystander cells in normal human cell cultures exposed to high LET radiations (Azzam, de Toledo et al. 1998; Azzam, de Toledo et al. 2001; Shao, Furusawa et al. 2003; Autsavapromporn, Suzuki et al. 2013). The results in Figure 3 (panel B) showing an increase in the level of $p21^{waf1}$, a cyclin-dependent kinase inhibitor, and connexin43, a major constituent of gap-junction, when AG1522 cell cultures are exposed to low fluence α particles under ambient oxygen atmosphere are consistent with previous results (Azzam, de Toledo et al. 2003; Autsavapromporn, de Toledo et al. 2011). At 0.1 cGy, less than 1 % of the cell nuclei were traversed by an α -particle track, yet $p21^{waf1}$ and connexin43 levels were increased by 2 and 2.1-fold, respectively. These increases were significantly reduced when the cell cultures were maintained and exposed to absorbed doses of α particles under low pO_2 . The up regulation observed under hypoxia at 0.3 cGy, when only 2.4 % of cell nuclei were traversed by an α particle track, although attenuated, was still twice as large compared to its own control.

DISCUSSION

Although in vitro radiation-induced bystander effects have been widely observed and the mechanisms underlying their expression are increasingly being understood, the spectrum of signaling events involved and their regulation at in vivo-like pO_2 is not yet clear. Several signaling pathways impacted by different modes of intercellular communication mediate radiation-induced bystander responses, and different cell types respond differently to biochemical signals exchanged among and between irradiated and bystander cells (Buonanno, Gonon et al. 2022). Notably, it has been suggested that reduced pO_2 elicits a variety of adaptive responses at both the tissue and cellular levels (Chandel and Schumacker 2000). These responses may greatly affect the nature and/or magnitude of the biological changes induced in bystander cells. Here, we provide further evidence that the redox environment, modulated by metabolic antioxidants, pO_2 , or treatment with prooxidants, plays a key role in the magnitude of the propagated radiation-induced bystander effects.

Glutathione peroxidase is an important antioxidant involved in the reduction of hydrogen peroxide and soluble lipid hydroperoxides that form as a result of normal oxidative metabolism and whose levels increase following cellular exposure to ionizing radiation (Burtcher, Mallet et al. 2022). Upregulating GPX expression by a genetic approach in bystander cells, protected them against oxidative stress propagated as a result of intimate co-culture with other cells having incorporated [3H]-thymidine (Figure 1). The Up-regulation of oxidative stress-responsive proteins (p21^{Waf1}, p-Hdm2, connexin43) in bystander cells expressing normal GPX levels was significantly reduced when GPX was ectopically overexpressed in bystander cells co-cultured with [3H]-thymidine-labelled

cells (Figure 1). These results support through rather direct approach that expression of bystander stressful effects is modulated by enzymes involved in oxidative metabolism as previously observed through treatment of cell cultures exposed to low fluences of α particles in presence or absence of antioxidant enzymes (Narayanan, Goodwin et al. 1997; Azzam, De Toledo et al. 2002). Under homeostatic conditions, GPX acts to maintain the balance between necessary and harmful levels of H_2O_2 and organic hydroperoxides (Handy and Loscalzo 2022). Previous results and those shown here show that redox sensitive processes culminating in an increase in prooxidant production that exceeds the ability of the cellular antioxidant capacity to maintain the normal steady state redox potential are tightly associated with the spread of oxidative stress from irradiated to bystander cells. The changes are consistent with involvement of a persistent perturbations in oxidative metabolism/activation of oxidase (Spitz, Azzam et al. 2004; Azzam, Jay-Gerin et al. 2012), as oxidative stress was observed in bystander cells not only in the hours and days following irradiation (Gonon, Groetz et al. 2013), but also in their progeny (Buonanno, de Toledo et al. 2011; de Toledo, Buonanno et al. 2017).

The effect of the redox environment on expression of bystander effects was further supported upon treatment of low fluence α particle-irradiated cultures with t-BOOH. Greater than expected increases in DNA damage as evaluated by micronuclei and 53BP1 foci formation was detected (Figure 2). Our earlier work under ambient pO_2 had shown that exposure of AG1522 cells to mean absorbed doses as low as 0.3 cGy of α particles activates as early as 1 min the redox-sensitive transcription factors NF- κ B, AP-1, ELK-1, p90-RSK and ATF2 (Azzam, De Toledo et al. 2002). Expansion of these studies to examine the expression of these transcription factors in normal cell cultures exposed to

low fluence α particles at in vivo-like pO_2 and when pO_2 is above ambient would further contribute to understanding the signaling pathways mediating bystander effects and would have relevance to radiation protection whether in occupational or clinical settings (Figure 4). Performing experiments in co-cultures of unirradiated normal cells and irradiated cancer cells, which notably have a prooxidant microenvironment (Klaunig, Wang et al. 2011; Gupta, Patel et al. 2014), would further inform on the impact of tissue pO_2 in propagation of bystander effects that contribute to normal tissue toxicity following radiotherapy. In the context of studies with α particle-emitters, it would further inform on recently described beneficial bystander effects leading to inhibition of proliferation and enhanced cell killing of bystander cancer cells in mice treated with targeted α emitters (Leung, Canter et al. 2020; Canter, Leung et al. 2021). It also has implications in treatment regimens where radiation is delivered in a fractionated manner. An enhancement of oxidative stress in bystander cells following delivery of the initial dose will greatly affect their response to subsequent doses. In particular, the modulating role of tissue pO_2 is fundamental to understanding the basic biological mechanisms involved in radiotherapeutic treatments at ultra-high dose rates (Adrian, Konradsson et al. 2020; Wilson, Hammond et al. 2020; Cao, Zhang et al. 2021).

Herein, we have further confirmed the expression of bystander effects in studies involving β particles emitted from tritium incorporated in DNA as well as external α particles (reviewed in (Buonanno, Gonon et al. 2022) (Figures 1-3). Notably, we show that cellular pO_2 modulates the magnitude of low fluence α particle-induced stressful bystander effects as measured by chromosomal damage (Figure 2) and changes in the expression levels of stress-responsive proteins (Figure 3). Whereas chromosomal damage

was significantly greater than expected at low doses delivered to the cell culture (1 cGy) at ~0.5 or ~1 % O₂, the damage was attenuated compared to measurements at 18.6 % O₂. The attenuation was greater when the cultures were exposed to 10 cGy rather than to lower doses, suggesting reduction of stress in both the irradiated and bystander cells as a greater fraction of cells are traversed by a particle track at 10 cGy (~80 % vs. ~2 at 0.2 cGy and ~8 % at 1 cGy) (Table 2).

Our earlier work had shown that ERK1/2 and other members of the redox sensitive mitogen activated protein kinase (MAPK) pathway are activated as early as 1 min after exposure of cell cultures to low doses of α particles (5 cGy) with the activation increasing in the span of 15 min during which measurements were performed (Azzam, De Toledo et al. 2002). Here we show that ERK1/2 activation measured at 3 h after exposure of cell cultures to 0.2, 1 or 10 cGy is greater under hypoxia than normoxia (Figure 3). Expansion of these studies to characterize downstream effectors of ERK1/2 and other members of the MAPK pathway (JNK, p38^{MAPK}) and their downstream effectors (e.g., ATF2, ELK-1, and AP-1) would enhance understating of the mediating signaling events. Interestingly, ERK1/2 phosphorylation was shown to be activated under reduced oxygen pressure in human microvascular endothelial cells-1 without increase in total ERK proteins, but with translocation to the nucleus (Richard, Berra et al. 1999; Minet, Arnould et al. 2000). Expansion of these studies to other cell types and examining the effect on the status of Hypoxia Inducible Factor 1 α (HIF-1 α) would be informative as HIF-1 α plays a major role in oxygen homeostasis regulation (Ziello, Jovin et al. 2007).

In earlier work, we generated direct evidence for the participation of gap junctional communication in the propagation of radiation-induced bystander effects

(Azzam, de Toledo et al. 2001). The intercellular channels that comprise gap junctions are formed by connexin proteins (Harris 2001), with connexin43 being highly abundant and a well-studied form in the connexin family (21 isoforms in humans). It is present in many tissues, including heart, central nervous system, liver, breast, skin, pancreas and lens (Laird 2006). The results reported here (Figure 3) showing differential regulation of connexin43 in bystander cells from cultures exposed to ionizing radiation at different pO_2 conditions suggests that intercellular communication depends on pO_2 , which likely impacts the overall response of cells/tissues to oxidative stress. Comparing the fold change in expression of connexin43 between skin fibroblast cultures irradiated at ~ 1 or 18.6 % O_2 , greater increases were detected 3 h after exposure to α particles at 18.6 % vs. ~ 1 % (Figure 3). The cellular distribution, phosphorylation state and levels of connexin43 in various types of cells were extensively studied following hypoxia/reoxygenation (Martinez and Saez 2000; Rodriguez-Sinovas, Garcia-Dorado et al. 2006; Marquez-Rosado, Solan et al. 2012) or chronic hypoxia as in obstructive sleep apnea syndrome (Kohutova, Elsnicova et al. 2019). Examining the effects of irradiation on redistribution of connexin43 within the cell (plasma membrane vs. cytoplasm), synthesis/degradation, and functional coupling between irradiated and bystander cells would enhance our mechanistic understanding of the role of junctional communication in bystander effects.

A decade after publication of the seminal paper by Nagasawa and Little on expression of cytogenetic changes in bystander cells (Nagasawa and Little 1992), a debate arose on the nature of the reactive species involved in triggering the spread of bystander effects (Ward 2002; Jay-Gerin, Meesungnoen et al. 2003). Follow up work to the early studies (Muroya, Plante et al. 2006) to characterize the identity as well as concentration of

reactive chemical species ($O_2^{\bullet-}$, H_2O_2 , $^{\bullet}NO$, etc.) generated in mammalian cells exposed to various types of radiation with different LET is warranted to enhance our understanding of the phenomenon and the early chemical/molecular signaling events that are involved (Hellweg 2015; Mukherjee, Dutta et al. 2021; Mukherjee, Dutta et al. 2022). Identifying these species in irradiated cells and the pathways they modulate at different pO_2 will be instructive of the dynamics of transition from early chemical events to subsequent biochemical changes induced in the irradiated cells and their subsequent propagation to bystander cells. In this context, it has been recently proposed that in presence of low concentrations of bicarbonate, the most significant oxidizing radical in cells is likely the carbonate anion radical, rather than the usually-invoked hydroxyl radical (Meyerstein 2022). Being a weaker oxidizing agent than the $OH^{\bullet}$ radical, $CO_3^{\bullet-}$ is more selective as a ROS (Meyerstein 2022). The use of dyes that detect specific reactive chemical species and bystander cells engineered to express redox sensitive transcription factors tagged to fluorescence proteins, coupled with real time microscopic observations, in identifiable irradiated and bystander cells, would inform on the kinetics of induction of redox modulated events.

In summary, the data reported in this study show by direct and indirect approaches that redox modulated events have a prominent role in the propagation of radiation-induced effects. They indicate that chromosomal damage and activation of signaling pathways (p53, MAPK, connexin expression) are sensitive to pO_2 changes. Based on our studies reported here and elsewhere and those of others (reviewed in (Buonanno, Gonon et al. 2022)), we propose that expression of radiation-induced bystander effects is dose-rate independent. Here, we showed that exposure of a cell culture to a very low fluence of

α particles (high LET radiation) induces events leading to propagation of oxidative stress from irradiated to non-irradiated cells. Likewise, co-culture of [^3H]-thymidine-labeled cells (low LET radiation) with bystander cells results in biochemical changes consistent with oxidative damage occurring in the bystander cells. In the α particle irradiation scenario, the irradiated cells are traversed by a single particle track (Table 2); therefore, a dose-rate effect is not involved. In contrast, in case of β particles from ^3H , the cells in the labelled culture are exposed at a high dose rate, which supports that in both cases, the physico-chemical and chemical steps are critical in induction of the observed bystander effects. Based on Monte-Carlo simulations of the track structure of densely and sparsely ionizing particles (Cobut, Frongillo et al. 1998; Frongillo, Goulet et al. 1998; Tippayamontri, Sunuchakan et al. 2009; Alanazi, Meesungnoen et al. 2020), whether traversed by a single α particle track or multiple electron tracks from ^3H , radical-radical combination/recombination reactions are favored due to the high density of energy deposition in the system. These interactions are favored within the high LET α particle track where the density of ionization events is extremely high, and in case of ^3H -labelled cells they occur between the low LET electron tracks whose number and spatial proximity as a function of time becomes very high due to delivery of the radiation at high dose rate. These patterns of increased intra- or inter-track radical-radical interactions facilitate ultimate formation of molecular products (e.g., H_2O_2) (Kuppermann 1961; Willis, Boyd et al. 1969; Pucheault, Ferradini et al. 1977; Jay-Gerin 2020; Alanazi, Meesungnoen et al. 2021; Sultana, Alanazi et al. 2022; Sultana, Alanazi et al. 2022). Whether the resulting DNA lesion(s) signaling induction of the bystander effect following exposure to low fluence α particles or to high dose rate β particles is similar remains to be investigated.

Studies involving analyses of single and double strand breaks as well as base damage in irradiated cells as well as the frequency and kinetics of occurrence of DNA damage foci would be informative.

Journal Pre-proof

ACKNOWLEDGMENTS

This work received support from the US National Institutes of Health (Grants CA92262 and CA049062), the Federal Nuclear Science and Technology program at Canadian Nuclear Laboratories (project FST-51320.0.A012), and the Natural Sciences and Engineering Research Council of Canada (Discovery Grant no. RGPIN-2022-03972). The authors thank Laura Bannister and Candice Didychuk for critical reading of the manuscript.

Disclosure of interest

The authors report no conflict of interest

TABLES

Table 1: Gaseous oxygen composition (in % and mm Hg) in dry air and in experimental atmosphere conditions, and its concentration in culture medium at 37° C.

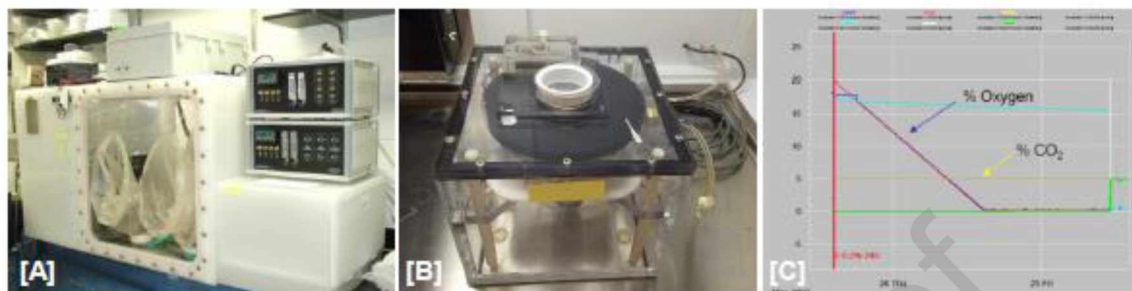
At sea level, the atmospheric pressure is 760 mm Hg. In a water saturated chamber maintained at 37°C, the partial pressure of water (pH₂O) is 47 mm Hg. In a classic incubator at ambient atmosphere (760 mm Hg), the partial pressure of 5 % carbon dioxide (pCO₂) represents 38 mm Hg. Therefore, 20.95 % of the remaining incubator air (760-47-38=675 mm Hg) results in pO₂ of 141 mm Hg. The latter pO₂ corresponds to 18.6 % O₂ (true normoxic oxygen condition) compared to ambient atmosphere (760 mm Hg). In our experiments, this value was decreased to respectively 0.88 % (6.7 mm Hg) and 0.44 % (3.3 mm Hg) in the two different hypoxic humidified conditions with 5 % CO₂. The concentrations of dissolved oxygen in culture medium maintained at a given temperature are proportional to the partial pressures of the gas above the solution according to Henry's law ($C = P/H$). At constant temperature of 37°C, the values corresponding to 18.6 %, 0.88 % and 0.44 % were respectively 182, 8.7, 4.3 µM. Henry's constant H of 771.65 mm Hg·mM⁻¹ was defined as a function of ionic strength of culture medium of 175 mM, which is an average value of most mammalian culture medium maintained without 5% CO₂ (Place, Domann et al. 2017). Numbers given in parentheses are the values in mm Hg.

	Ambient dry air	Standard incubator (humidified)	Hypoxic incubator (humidified)	
			~ 1%	~ 0.5%
H ₂ O _g	-	6.2% (47)	6.2% (47)	6.2% (47)
CO ₂	0.04% (0.3)	5% (38)	5% (38)	5% (38)
O ₂	20.95% (159)	18.6% (141)	0.88% (6.7)	0.44% (3.3)
Dissolved O ₂ in medium		182 µM	8.7 µM	4.3 µM
N ₂	79.01% (600)	70.2% (534)	87.92% (668)	88.36% (672)

Table 2: Energy and linear energy transfer (LET) of α particles and estimates of α particle traversals in confluent AG1522 cell cultures exposed to an absorbed dose of 0.1, 0.2, 0.3, 1 or 10 cGy. The fluence (particles/cm²) is given by the following relation ϕ (particles/cm²) = [D (cGy) · ρ (g/cm³)]/[1.602 × 10⁻⁷ · LET (keV/μm)], where D is the mean dose ρ , the density. The average number of nucleus and cell particle traversals was established on the average cross section of an AG1522 cell nucleus [i.e., 140 μm²] or with the average cross-sectional area of an AG1522 whole cell [i.e., 800 μm²] as described in (Gonon, Groetz et al. 2013). Estimates of the fractions of whole cells or nuclei traversed by a α particle were determined according to the method of Charlton and Sephton (Charlton and Sephton 1991). Assuming Poisson statistics, the probability P that a given target area is traversed by N particles is given by $P(N) = e^{-x} x^N / N!$ with x being the product of the fluence and the target cross-sectional area (nucleus or whole cell).

	Energy (MeV/u)	LET (keV/μm)	Dose (cGy)	Fluence (part./cm ²)	Average number of traversals		Fraction of cells' nuclei traversed by 0, 1 or more than 2 particles		
					Cell	Nucleus			
					Avg.	Avg.	P(0)	P(1)	P(≥2)
⁴ He ²⁺ (α)	0.92	109	0.1	5.7 × 10 ³	0.046	0.008	0.992	0.008	0.000
			0.2	1.1 × 10 ⁴	0.092	0.016	0.984	0.016	0.000
			0.3	1.7 × 10 ⁴	0.138	0.024	0.976	0.024	0.000
			1.0	5.7 × 10 ⁴	0.458	0.080	0.923	0.074	0.003
			10	5.7 × 10 ⁵	4.580	0.802	0.449	0.360	0.191

SCHEMATIC



Schematic 1: (A) Custom-made 'Xvivo' chamber equipped with two incubators whose O₂ and CO₂ atmosphere are independently adjustable; (B) the α particle irradiator located in the work space of the chamber; (C) traces describing the decrease in dissolved oxygen concentration (red colour) from ambient conditions to a hypoxic environment (here as low as 0.2 %) over a 24 h period. Light blue and green colour traces refer, respectively, to the percent O₂ and percent CO₂ in the control incubator environment. Dark blue and yellow traces refer, respectively, to the percent oxygen and percent CO₂ in the experimental incubator environment. Upon reaching the desired dissolved oxygen concentration, the cell cultures were maintained for an additional 24 h at the desired concentration before irradiation. The CO₂ concentration was maintained at 5% throughout. The cell cultures were irradiated at the respective O₂ and CO₂ atmosphere in the workspace and maintained in the same atmosphere for the desired time following irradiation.

FIGURE LEGENDS

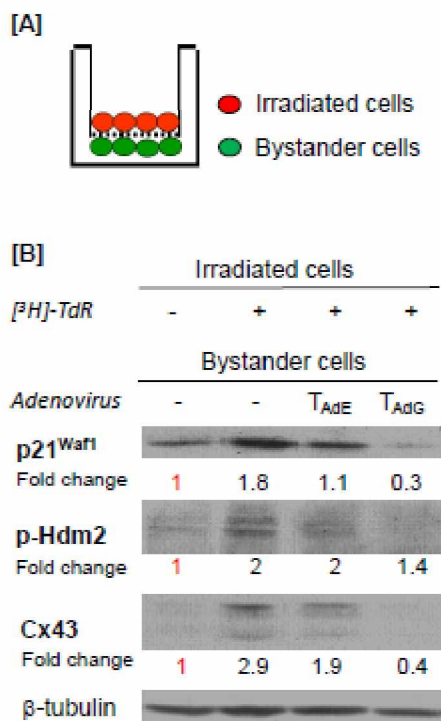


Figure 1: Effect of ectopic over-expression of glutathione peroxidase on expression levels of stress responsive proteins in bystander AG1522 cells. (A) Schematic of co-culture strategy; (B) Western blot analyses in bystander AG1522 cells co-cultured for 6 h with other AG1522 cells that have proliferated in growth medium containing 37 kBq/ml [³H]-thymidine. The bystander cells were either maintained at homeostatic genomic conditions or were transduced with either empty vector (T_{AdE}) or a vector incorporating the human gene encoding GLUTATHIONE PEROXIDASE (T_{AdG}). Reaction with anti-β-tubulin antibody was used as the loading control. Fold change represents relative change compared to the control indicated in red.

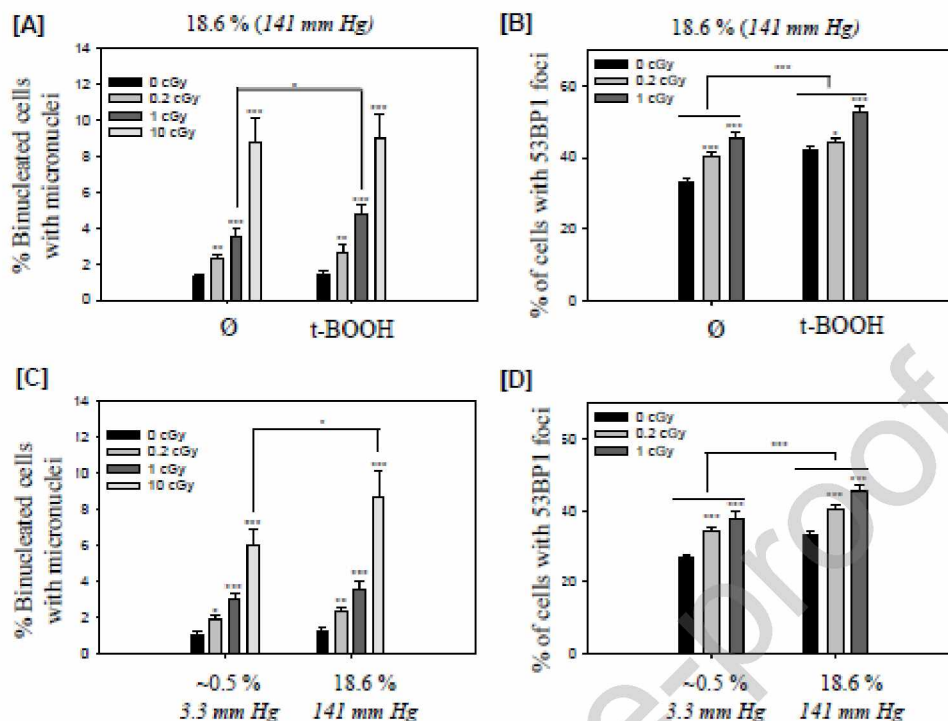


Figure 2: Effect of the redox environment on chromosomal damage on bystander responses: effect of pro-oxidant environment and of partial oxygen pressure. Percentage of binucleated cells harbouring micronuclei (panels A and C) and percentage of cell presenting 53BP1 foci (panels B and D) in confluent AG1522 cell cultures 3 h after exposure to an absorbed dose of 0, 0.2, 1 or 10 cGy from 3.7 MeV α particles. The cell cultures were maintained at 18.6 % oxygen atmosphere in presence or absence of 0.5 μ M t-butyl hydroperoxide 1 h before irradiation (panels A and B) or at different oxygen atmosphere (~0.5 or 18.6 %) 48 h before irradiation (panels C and D). They were maintained in the same culture conditions until harvest time, 3 h after irradiation. The results obtained under an atmosphere of 18.6 % O₂ in absence of the drug (left side in panels A and B) are the same results described, respectively, in panels C and D (right side) as all experiments were performed in parallel. For micronucleus assay, the cell cultures were subcultured in presence of cytochalasin B 3 h after exposure. (*: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$)

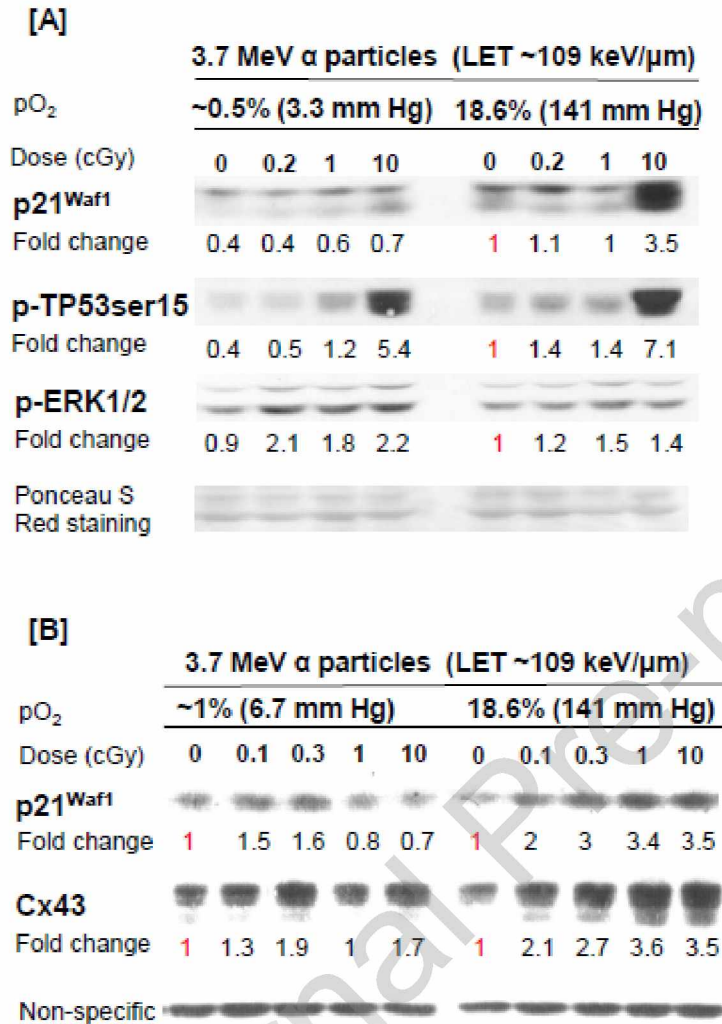


Figure 3: The effect of partial oxygen pressure (pO₂) in propagation of radiation-induced bystander effects. Western blot analyses of stress responsive proteins (p21^{Waf1}, p-TP53ser15, p-ERK1/2, Cx43) in confluent normal human AG1522 cell cultures 3 h after exposure to low fluences of α particles (absorbed dose of 0, 0.1, 0.2, 0.3, 1 or 10 cGy) under ambient (18.6% O₂) or in vivo-like pO₂ (panel A: ~0.5% O₂; panel B: ~1% O₂). Staining with Ponceau S Red or non-specific antibody reaction was used as the loading control. Each immunoblot is representative of 3 experiments. Fold change represents relative change compared to the control (i.e., 0 cGy) indicated in red.

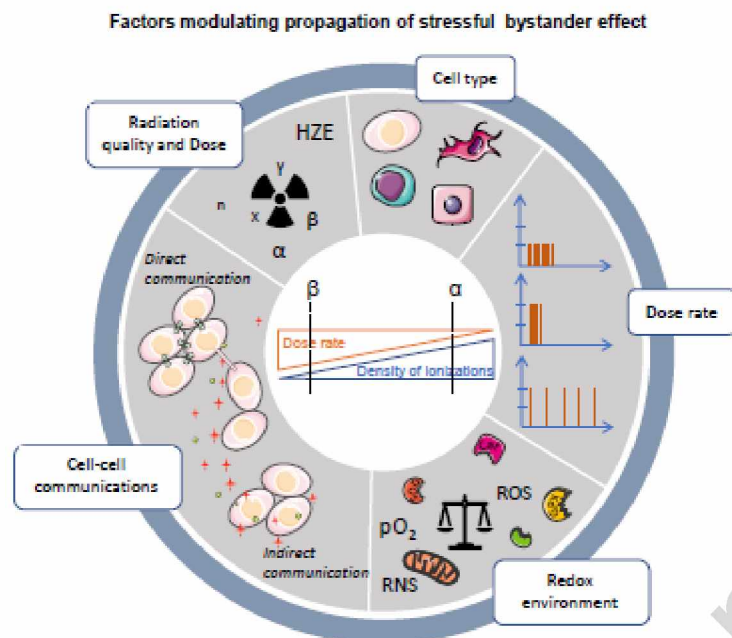


Figure 4: Factors modulating propagation of ionizing radiation-induced stressful bystander effect: role of the redox environment, radiation quality and dose, radiation dose-rate, cell type, and the intercellular communications involved. The image elements are not to scale with each other. Images were modified using Servier Medical Art by Servier which is licensed under a Creative Commons Attribution 3.0 Unported License <https://smart.servier.com/>.

ROS: reactive oxygen species; RNS: reactive nitrogen species; Cat: Catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase; HZE: High atomic number (Z) and high energy (E) particles

Cell-cell communications may involve gap junction, nanotubes, hemichannels, pannexins, and secreted factors (e.g. soluble molecules, exosomes, micro-vesicles).

References

- Adrian, G., E. Konradsson, et al. (2020). "The FLASH effect depends on oxygen concentration." *Br J Radiol* **93**(1106): 20190702.
- Alanazi, A., J. Meesungnoen, et al. (2020). "Linear energy transfer dependence of transient yields in water irradiated by 150 keV – 500 MeV protons in the limit of low dose rates." *Canadian Journal of Chemistry* **98**(8): 427-433.
- Alanazi, A., J. Meesungnoen, et al. (2021). "A Computer Modeling Study of Water Radiolysis at High Dose Rates. Relevance to FLASH Radiotherapy." *Radiat Res* **195**(2): 149-162.
- Alper, T. and P. E. Bryant (1974). "Reduction in oxygen enhancement ratio with increase in LET: tests of two hypotheses." *Int J Radiat Biol Relat Stud Phys Chem Med* **26**(3): 203-218.
- Asaithamby, A., N. Uematsu, et al. (2008). "Repair of HZE-particle-induced DNA double-strand breaks in normal human fibroblasts." *Radiat Res* **169**(4): 437-446.
- Ast, T. and V. K. Mootha (2019). "Oxygen and mammalian cell culture: are we repeating the experiment of Dr. Ox?" *Nat Metab* **1**(9): 858-860.
- Autsavapromporn, N., S. M. de Toledo, et al. (2011). "The role of gap junction communication and oxidative stress in the propagation of toxic effects among high-dose alpha-particle-irradiated human cells." *Radiat Res* **175**(3): 347-357.
- Autsavapromporn, N., M. Suzuki, et al. (2013). "Gap junction communication and the propagation of bystander effects induced by microbeam irradiation in human fibroblast cultures: the impact of radiation quality." *Radiat Res* **180**(4): 367-375.
- Azzam, E. I., S. M. de Toledo, et al. (1998). "Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles." *Radiat Res* **150**(5): 497-504.
- Azzam, E. I., S. M. de Toledo, et al. (2001). "Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells." *Proc Natl Acad Sci U S A* **98**(2): 473-478.
- Azzam, E. I., S. M. de Toledo, et al. (2003). "Expression of CONNEXIN43 is highly sensitive to ionizing radiation and other environmental stresses." *Cancer Res* **63**(21): 7128-7135.
- Azzam, E. I., S. M. de Toledo, et al. (2003). "Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect." *Oncogene* **22**(45): 7050-7057.
- Azzam, E. I., S. M. De Toledo, et al. (2002). "Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures." *Cancer Res* **62**(19): 5436-5442.
- Azzam, E. I., J. P. Jay-Gerin, et al. (2012). "Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury." *Cancer Lett* **327**(1-2): 48-60.
- Bambrick, L. L., Y. Kostov, et al. (2011). "In vitro cell culture pO₂ is significantly different from incubator pO₂." *Biotechnol Prog* **27**(4): 1185-1189.
- Barendsen, G. W., C. J. Koot, et al. (1966). "The effect of oxygen on impairment of the proliferative capacity of human cells in culture by ionizing radiations of different LET." *Int J Radiat Biol Relat Stud Phys Chem Med* **10**(4): 317-327.

- Baverstock, K. F. and W. G. Burns (1981). "Oxygen as a product of water radiolysis in high-LET tracks. II. Radiobiological implications." *Radiat Res* **86**(1): 20-33.
- Bedford, J. S. and J. B. Mitchell (1974). "The effect of hypoxia on the growth and radiation response of mammalian cells in culture." *Br J Radiol* **47**(562): 687-696.
- Brown, J. M. and A. J. Giaccia (1994). "Tumour hypoxia: the picture has changed in the 1990s." *Int J Radiat Biol* **65**(1): 95-102.
- Browne, E., R. B. Firestone, et al. (1986). Table of radioactive isotopes. United States, John Wiley and Sons Inc.
- Bunn, H. F. and R. O. Poyton (1996). "Oxygen sensing and molecular adaptation to hypoxia." *Physiol Rev* **76**(3): 839-885.
- Buonanno, M., S. M. de Toledo, et al. (2011). "Long-term consequences of radiation-induced bystander effects depend on radiation quality and dose and correlate with oxidative stress." *Radiat Res* **175**(4): 405-415.
- Buonanno, M., G. Gonon, et al. (2022). "The intercellular communications mediating radiation-induced bystander effects and their relevance to environmental, occupational, and therapeutic exposures." *Int J Radiat Biol*: 1-19.
- Burtscher, J., R. T. Mallet, et al. (2022). "Adaptive Responses to Hypoxia and/or Hyperoxia in Humans." *Antioxid Redox Signal*.
- Canter, B. S., C. N. Leung, et al. (2021). "Radium-223-Induced Bystander Effects Cause DNA Damage and Apoptosis in Disseminated Tumor Cells in Bone Marrow." *Mol Cancer Res* **19**(10): 1739-1750.
- Cao, X., R. Zhang, et al. (2021). "Quantification of Oxygen Depletion During FLASH Irradiation In Vitro and In Vivo." *Int J Radiat Oncol Biol Phys* **111**(1): 240-248.
- Carreau, A., B. El Hafny-Rahbi, et al. (2011). "Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia." *J Cell Mol Med* **15**(6): 1239-1253.
- Chandel, N. S., E. Maltepe, et al. (1998). "Mitochondrial reactive oxygen species trigger hypoxia-induced transcription." *Proc Natl Acad Sci U S A* **95**(20): 11715-11720.
- Chandel, N. S., D. S. McClintock, et al. (2000). "Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 α during hypoxia: a mechanism of O₂ sensing." *J Biol Chem* **275**(33): 25130-25138.
- Chandel, N. S. and P. T. Schumacker (2000). "Cellular oxygen sensing by mitochondria: old questions, new insight." *J Appl Physiol* **88**(5): 1880-1889.
- Charlton, D. E. and R. Sephton (1991). "A relationship between microdosimetric spectra and cell survival for high-LET irradiation." *Int J Radiat Biol* **59**(2): 447-457.
- Clanton, T. L. (2007). "Hypoxia-induced reactive oxygen species formation in skeletal muscle." *J Appl Physiol* **102**(6): 2379-2388.
- Cobut, V., Y. Frongillo, et al. (1998). "Monte Carlo simulation of fast electron and proton tracks in liquid water -- I. physical and physicochemical aspects." *Radiation Physics and Chemistry* **51**: 229-243.
- de Toledo, S. M., N. Asaad, et al. (2006). "Adaptive responses to low-dose/low-dose-rate gamma rays in normal human fibroblasts: the role of growth architecture and oxidative metabolism." *Radiat Res* **166**(6): 849-857.
- de Toledo, S. M., M. Buonanno, et al. (2017). "Genomic instability induced in distant progeny of bystander cells depends on the connexins expressed in the irradiated cells." *Int J Radiat Biol* **93**(10): 1182-1194.

- Denault, C. M. and H. L. Liber (1993). "The effects of hypoxia and cysteamine on X-ray mutagenesis in human cells. I. Dose response and Southern blot analysis of induced mutants." *Radiat Res* **135**(1): 98-107.
- Domogauer, J. D., S. M. de Toledo, et al. (2016). "A Mimic of the Tumor Microenvironment: A Simple Method for Generating Enriched Cell Populations and Investigating Intercellular Communication." *J Vis Exp*(115).
- Fenech, M. and A. A. Morley (1985). "Measurement of micronuclei in lymphocytes." *Mutat Res* **147**(1-2): 29-36.
- Frankenberg-Schwager, M., D. Frankenberg, et al. (1991). "Different oxygen enhancement ratios for induced and unrejoined DNA double-strand breaks in eukaryotic cells." *Radiat Res* **128**(3): 243-250.
- Frongillo, Y., T. Goulet, et al. (1998). "Monte Carlo simulation of fast electron and proton tracks in liquid water -- II. nonhomogeneous chemistry." *Radiation Physics and Chemistry* **51**: 245-254.
- Fuciarelli, A. F., B. J. Wegher, et al. (1990). "Yields of radiation-induced base products in DNA: effects of DNA conformation and gassing conditions." *Int J Radiat Biol* **58**(3): 397-415.
- Gerashchenko, B. I. and R. W. Howell (2004). "Proliferative response of bystander cells adjacent to cells with incorporated radioactivity." *Cytometry A* **60**(2): 155-164.
- Gerashchenko, B. I. and R. W. Howell (2005). "Bystander cell proliferation is modulated by the number of adjacent cells that were exposed to ionizing radiation." *Cytometry A* **66**(1): 62-70.
- Giaccia, A. J., M. C. Simon, et al. (2004). "The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease." *Genes Dev* **18**(18): 2183-2194.
- Goddu, S. M., R. W. Howell, et al. (1994). "Cellular dosimetry: absorbed fractions for monoenergetic electron and alpha particle sources and S-values for radionuclides uniformly distributed in different cell compartments." *J Nucl Med* **35**(2): 303-316.
- Gonon, G., J. E. Groetz, et al. (2013). "Nontargeted stressful effects in normal human fibroblast cultures exposed to low fluences of high charge, high energy (HZE) particles: kinetics of biologic responses and significance of secondary radiations." *Radiat Res* **179**(4): 444-457.
- Graeber, T. G., J. F. Peterson, et al. (1994). "Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status." *Mol Cell Biol* **14**(9): 6264-6277.
- Gray, L. H., A. D. Conger, et al. (1953). "The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy." *Br J Radiol* **26**(312): 638-648.
- Grdina, D. J., B. Nagy, et al. (1989). "Protection against radiation-induced mutagenesis in V79 cells by 2-[(aminopropyl)amino] ethanethiol under conditions of acute hypoxia." *Radiat Res* **117**(2): 251-258.
- Guidarelli, A., F. Cattabeni, et al. (1997). "Alternative mechanisms for hydroperoxide-induced DNA single strand breakage." *Free Radic Res* **26**(6): 537-547.
- Gupta, R. K., A. K. Patel, et al. (2014). "Oxidative stress and antioxidants in disease and cancer: a review." *Asian Pac J Cancer Prev* **15**(11): 4405-4409.

- Hamm, R. N., J. E. Turner, et al. (1985). "Calculation of heavy-ion tracks in liquid water." *Radiat Res Suppl* **8**: S20-26.
- Handy, D. E. and J. Loscalzo (2022). "The role of glutathione peroxidase-1 in health and disease." *Free Radic Biol Med* **188**: 146-161.
- Harris, A. L. (2001). "Emerging issues of connexin channels: biophysics fills the gap." *Q Rev Biophys* **34**(3): 325-472.
- Hei, T. K., H. Zhou, et al. (2011). "Radiation Induced Non-targeted Response: Mechanism and Potential Clinical Implications." *Curr Mol Pharmacol* **4**(2): 96-105.
- Held, K. D., G. D. Bren, et al. (1986). "Interactions of radioprotectors and oxygen in cultured mammalian cells. II. Effects of dithiothreitol on radiation-induced DNA damage and comparison with cell survival." *Radiat Res* **108**(3): 296-306.
- Hellweg, C. E. (2015). "The Nuclear Factor kappaB pathway: A link to the immune system in the radiation response." *Cancer Lett* **368**(2): 275-289.
- Jagannathan, L., S. Cuddapah, et al. (2016). "Oxidative stress under ambient and physiological oxygen tension in tissue culture." *Curr Pharmacol Rep* **2**(2): 64-72.
- Jay-Gerin, J. P. (2020). "Ultra-high dose-rate (FLASH) radiotherapy: Generation of early, transient, strongly acidic spikes in the irradiated tumor environment." *Cancer Radiother* **24**(4): 332-334.
- Jay-Gerin, J. P., J. Meesungnoen, et al. (2003). "Comment on "The radiation-induced lesions which trigger the bystander effect" by J.F. Ward [Mutat. Res. 499 (2002) 151-154]." *Mutat Res* **525**(1-2): 125-127.
- Kaplan, A. R. and P. M. Glazer (2020). "Impact of hypoxia on DNA repair and genome integrity." *Mutagenesis* **35**(1): 61-68.
- Keeley, T. P. and G. E. Mann (2019). "Defining Physiological Normoxia for Improved Translation of Cell Physiology to Animal Models and Humans." *Physiol Rev* **99**(1): 161-234.
- Kim, J. A., Y. S. Kang, et al. (1998). "Role of Ca²⁺ influx in the tert-butyl hydroperoxide-induced apoptosis of HepG2 human hepatoblastoma cells." *Exp Mol Med* **30**(3): 137-144.
- Klaunig, J. E., Z. Wang, et al. (2011). "Oxidative stress and oxidative damage in chemical carcinogenesis." *Toxicol Appl Pharmacol* **254**(2): 86-99.
- Kohutova, J., B. Elsnicova, et al. (2019). "Anti-arrhythmic Cardiac Phenotype Elicited by Chronic Intermittent Hypoxia Is Associated With Alterations in Connexin-43 Expression, Phosphorylation, and Distribution." *Frontiers in Endocrinology* **9**.
- Kuppermann, A. (1961). Diffusion kinetics in radiation chemistry. *Actions Chimiques et Biologiques des Radiations*. Paris, Masson: 85-166.
- Laird, D. W. (2006). "Life cycle of connexins in health and disease." *Biochem J* **394**(Pt 3): 527-543.
- Lawrence, R. A. and R. F. Burk (1976). "Glutathione peroxidase activity in selenium-deficient rat liver." *Biochem Biophys Res Commun* **71**(4): 952-958.
- Lehnert, B. E., E. H. Goodwin, et al. (1997). "Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells." *Cancer Res* **57**(11): 2164-2171.

- Leung, C. N., B. S. Canter, et al. (2020). "Dose-Dependent Growth Delay of Breast Cancer Xenografts in the Bone Marrow of Mice Treated with (223)Ra: The Role of Bystander Effects and Their Potential for Therapy." *J Nucl Med* **61**(1): 89-95.
- Limon-Pacheco, J. and M. E. Gonsbatt (2009). "The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress." *Mutat Res* **674**(1-2): 137-147.
- Little, J. B. (2003). "Genomic instability and bystander effects: a historical perspective." *Oncogene* **22**(45): 6978-6987.
- Lopez-Barneo, J., J. R. Lopez-Lopez, et al. (1988). "Chemotransduction in the carotid body: K⁺ current modulated by PO₂ in type I chemoreceptor cells." *Science* **241**(4865): 580-582.
- Lowry, O. H., N. J. Rosebrough, et al. (1951). "Protein measurement with the Folin phenol reagent." *J Biol Chem* **193**(1): 265-275.
- Marquez-Rosado, L., J. L. Solan, et al. (2012). "Connexin43 phosphorylation in brain, cardiac, endothelial and epithelial tissues." *Biochim Biophys Acta* **1818**(8): 1985-1992.
- Martinez, A. D. and J. C. Saez (2000). "Regulation of astrocyte gap junctions by hypoxia-reoxygenation." *Brain Res Brain Res Rev* **32**(1): 250-258.
- Meesungnoen, J. and J. P. Jay-Gerin (2009). "High-LET ion radiolysis of water: oxygen production in tracks." *Radiat Res* **171**(3): 379-386.
- Meyerstein, D. (2022). "What Are the Oxidizing Intermediates in the Fenton and Fenton-like Reactions? A Perspective." *Antioxidants* **11**(7).
- Michiels, C. (2004). "Physiological and pathological responses to hypoxia." *Am J Pathol* **164**(6): 1875-1882.
- Minet, E., T. Arnould, et al. (2000). "ERK activation upon hypoxia: involvement in HIF-1 activation." *FEBS Lett* **468**(1): 53-58.
- Mothersill, C. and C. B. Seymour (2004). "Radiation-induced bystander effects--implications for cancer." *Nat Rev Cancer* **4**(2): 158-164.
- Mukherjee, S., A. Dutta, et al. (2021). "External modulators and redox homeostasis: Scenario in radiation-induced bystander cells." *Mutation Research/Reviews in Mutation Research* **787**: 108368.
- Mukherjee, S., A. Dutta, et al. (2022). "The interaction of oxidative stress with MAPK, PI3/AKT, NF- κ B, and DNA damage kinases influences the fate of γ -radiation-induced bystander cells." *Archives of Biochemistry and Biophysics* **725**: 109302.
- Muroya, Y., I. Plante, et al. (2006). "High-LET ion radiolysis of water: visualization of the formation and evolution of ion tracks and relevance to the radiation-induced bystander effect." *Radiat Res* **165**(4): 485-491.
- Nagasawa, H. and J. B. Little (1992). "Induction of sister chromatid exchanges by extremely low doses of alpha-particles." *Cancer Res* **52**(22): 6394-6396.
- Nakayama, K. and N. Kataoka (2019). "Regulation of Gene Expression under Hypoxic Conditions." *Int J Mol Sci* **20**(13).
- Narayanan, P. K., E. H. Goodwin, et al. (1997). "Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells." *Cancer Res* **57**(18): 3963-3971.

- Neti, P. V., S. M. de Toledo, et al. (2004). "A multi-port low-fluence alpha-particle irradiator: fabrication, testing and benchmark radiobiological studies." *Radiat Res* **161**(6): 732-738.
- Petry, E. (1923). "Zur kenntnis der bedingungen der biologischen wirkung der rontgenstrahlen." *Biochem. Zeitschr* **135**: 353.
- Place, T. L., F. E. Domann, et al. (2017). "Limitations of oxygen delivery to cells in culture: An underappreciated problem in basic and translational research." *Free Radic Biol Med* **113**: 311-322.
- Prabhakar, N. R. and G. L. Semenza (2015). "Oxygen Sensing and Homeostasis." *Physiology (Bethesda)* **30**(5): 340-348.
- Prise, K. M., M. Folkard, et al. (1990). "The irradiation of V79 mammalian cells by protons with energies below 2 MeV. Part II. Measurement of oxygen enhancement ratios and DNA damage." *Int J Radiat Biol* **58**(2): 261-277.
- Prise, K. M., M. Folkard, et al. (2003). "A review of the bystander effect and its implications for low-dose exposure." *Radiat Prot Dosimetry* **104**(4): 347-355.
- Pucheault, J., C. Ferradini, et al. (1977). "Fast kinetic studies of chemical reactions using high-intensity pulse radiolysis reaction of hydrogen peroxide with pentavalent vanadium." *International Journal of Chemical Kinetics* **9**(4): 603-617.
- Rappold, I., K. Iwabuchi, et al. (2001). "Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways." *J Cell Biol* **153**(3): 613-620.
- Richard, D. E., E. Berra, et al. (1999). "p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1." *J Biol Chem* **274**(46): 32631-32637.
- Rodriguez-Sinovas, A., D. Garcia-Dorado, et al. (2006). "Protective effect of gap junction uncouplers given during hypoxia against reoxygenation injury in isolated rat hearts." *Am J Physiol Heart Circ Physiol* **290**(2): H648-656.
- Romero-Calvo, I., B. Ocon, et al. (2010). "Reversible Ponceau staining as a loading control alternative to actin in Western blots." *Anal Biochem* **401**(2): 318-320.
- Russo, A., J. Mitchell, et al. (1985). "Determinants of radiosensitivity." *Semin Oncol* **12**(3): 332-349.
- Sandstrom, B. E. (1991). "Induction and rejoining of DNA single-strand breaks in relation to cellular growth in human cells exposed to three hydroperoxides at 0 degrees C and 37 degrees C." *Free Radic Res Commun* **15**(2): 79-89.
- Schultz, L. B., N. H. Chehab, et al. (2000). "p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks." *J Cell Biol* **151**(7): 1381-1390.
- Semenza, G. L. (2000). "HIF-1: mediator of physiological and pathophysiological responses to hypoxia." *J Appl Physiol* **88**(4): 1474-1480.
- Shao, C., Y. Furusawa, et al. (2003). "Role of gap junctional intercellular communication in radiation-induced bystander effects in human fibroblasts." *Radiat Res* **160**(3): 318-323.
- Shao, C., Y. Furusawa, et al. (2003). "Bystander effect induced by counted high-LET particles in confluent human fibroblasts: a mechanistic study." *FASEB J* **17**(11): 1422-1427.
- Shimoda, L. A. and J. Polak (2011). "Hypoxia. 4. Hypoxia and ion channel function." *Am J Physiol Cell Physiol* **300**(5): C951-967.

- Sorensen, B. S. and M. R. Horsman (2020). "Tumor Hypoxia: Impact on Radiation Therapy and Molecular Pathways." *Front Oncol* **10**: 562.
- Spitz, D. R., E. I. Azzam, et al. (2004). "Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology." *Cancer Metastasis Rev* **23**(3-4): 311-322.
- Sultana, A., A. Alanazi, et al. (2022). "Generation of ultrafast, transient, highly acidic pH spikes in the radiolysis of water at very high dose rates: relevance for FLASH radiotherapy." *Canadian Journal of Chemistry* **100**(4): 272-279.
- Sultana, A., A. Alanazi, et al. (2022). "On the Transient Radiolytic Oxygen Depletion in the Ultra-High (FLASH) Dose-Rate Radiolysis of Water in a Cell-Like Environment: Effect of e-aq and *OH Competing Scavengers." *Radiat Res* **197**(5): 566-567.
- Tippayamontri, T., S. Sunuchakan, et al. (2009). Fast neutron radiolysis of the ferrous sulfate (Fricke) dosimeter: Monte Carlo simulations. *Recent Research Developments in Physical Chemistry*. S. G. Pandalai, Ed. Trivandrum, Kerala, India. **10**: 143-211.
- Tisljar-Lentulis, G., P. Henneberg, et al. (1983). "The oxygen enhancement ratio for single- and double-strand breaks induced by tritium incorporated in DNA of cultured human T1 cells. Impact of the transmutation effect." *Radiat Res* **94**(1): 41-50.
- Ward, J. F. (2002). "The radiation-induced lesions which trigger the bystander effect." *Mutat Res* **499**(2): 151-154.
- Wenger, R. H., V. Kurtcuoglu, et al. (2015). "Frequently asked questions in hypoxia research." *Hypoxia (Auckl)* **3**: 35-43.
- Willis, C., A. W. Boyd, et al. (1969). "Experimental and calculated yields in the radiolysis of water at very high dose rates." *International Journal for Radiation Physics and Chemistry* **1**(3): 373-381.
- Wilson, J. D., E. M. Hammond, et al. (2020). "Ultra-High Dose Rate (FLASH) Radiotherapy: Silver Bullet or Fool's Gold?" *Frontiers in Oncology* **9**.
- Wilson, P. F., P. B. Nham, et al. (2010). "Inter-individual variation in DNA double-strand break repair in human fibroblasts before and after exposure to low doses of ionizing radiation." *Mutat Res* **683**(1-2): 91-97.
- Wilson, R. E. and R. M. Sutherland (1989). "Enhanced synthesis of specific proteins, RNA, and DNA caused by hypoxia and reoxygenation." *Int J Radiat Oncol Biol Phys* **16**(4): 957-961.
- Woodbine, L., H. Brunton, et al. (2011). "Endogenously induced DNA double strand breaks arise in heterochromatic DNA regions and require ataxia telangiectasia mutated and Artemis for their repair." *Nucleic Acids Res* **39**(16): 6986-6997.
- Zakaria, A. M., N. W. Colangelo, et al. (2020). "Ultra-High Dose-Rate, Pulsed (FLASH) Radiotherapy with Carbon Ions: Generation of Early, Transient, Highly Oxygenated Conditions in the Tumor Environment." *Radiat Res* **194**(6): 587-593.
- Zhang, J., Y. Zhang, et al. (2021). "The Roles of HIF-1 α in Radiosensitivity and Radiation-Induced Bystander Effects Under Hypoxia." *Front Cell Dev Biol* **9**: 637454.
- Zhou, H., G. Randers-Pehrson, et al. (2000). "Induction of a bystander mutagenic effect of alpha particles in mammalian cells." *Proc Natl Acad Sci U S A* **97**(5): 2099-2104.

- Zhou, H., M. Suzuki, et al. (2001). "Radiation risk to low fluences of alpha particles may be greater than we thought." *Proc Natl Acad Sci U S A* **98**(25): 14410-14415.
- Ziello, J. E., I. S. Jovin, et al. (2007). "Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia." *Yale J Biol Med* **80**(2): 51-60.

Journal Pre-proof

Highlights

- Radiation-induced bystander effects are attenuated at below ambient pO_2 .
- A prooxidant cellular environment amplifies radiation bystander effects.
- Early chemical steps impact the radiation bystander response.
- The bystander effect is independent of radiation dose rate.
- The reported results are relevant to radiation protection and radiotherapy.