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Research Paper

Characterizing low dose and dose rate effects in rodent and human neural stem cells exposed to proton and gamma irradiation

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ABSTRACT

Past work has shown that exposure to gamma rays and protons elicit a persistent oxidative stress in rodent and human neural stem cells (hNSCs). We have now adapted these studies to more realistic exposure scenarios in space, using lower doses and dose rates of these radiation modalities, to further elucidate the role of radiation-induced oxidative stress in these cells. Rodent neural stem and precursor cells grown as neurospheres and human neural stem cells grown as monolayers were subjected to acute and multi-dosing paradigms at differing dose rates and analyzed for changes in reactive oxygen species (ROS), reactive nitrogen species (RNS), nitric oxide and superoxide for 2 days after irradiation. While acute exposures led to significant changes in both cell types, hNSCs in particular, exhibited marked and significant elevations in radiation-induced oxidative stress. Elevated oxidative stress was more significant in hNSCs as opposed to their rodent counterparts, and hNSCs were significantly more sensitive to low dose exposures in terms of survival. Combinations of protons and γ -rays delivered as lower priming or higher challenge doses elicited radioadaptive changes that were associated with improved survival, but in general, only under conditions where the levels of reactive species were suppressed compared to cells irradiated acutely. Protective radioadaptive effects on survival were eliminated in the presence of the antioxidant N-acetylcysteine, suggesting further that radiation-induced oxidative stress could activate pro-survival signaling pathways that were sensitive to redox state. Data corroborates much of our past work and shows that low dose and dose rate exposures elicit significant changes in oxidative stress that have functional consequences on survival.

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Introduction

The space radiation environment is characterized by a range of energetic charged particles predominated by protons, derived from a variety of solar events, present in trapped radiation belts around the earth, and highly represented within the spectrum of particles defining galactic cosmic rays (GCR) [1]. Protons from each of these sources have characteristic fluences and energies ranging from several MeV/n to GeV/n, and can contribute to the

absorbed dose over the duration of any mission into space [1]. However, even when higher exposure scenarios are considered, such doses are not projected to exceed 2 Gy, as most cellular traversals involving protons would not be more frequent than daily occurrences [2,3]. Thus, the reality of the space radiation environment suggests the need to focus studies at lower total doses and dose rates, in efforts to provide more meaningful information relevant to risk estimates [4,5].

Past work from our lab has characterized the radioresponse of rodent and human neural stem cells (hNSCs) exposed to both low and higher LET radiations [6–8]. These studies have shown that cells exposed to a range of biologically relevant doses exhibit an acute and persistent radiation-induced oxidative stress. The resultant oxidative stress has in general been found to be dose-responsive and dependent upon radiation quality, where it impacts the survival and differentiation of cells both in vitro and in vivo [7–10]. How oxidative stress impacts the functionality of neural stem cells also depends upon the nature and duration of that particular reactive species. Chronic hydrogen peroxide has been found to sensitize neural stem and precursor cells in culture to the action of ionizing radiation [11], but excess superoxide had

Abbreviations: ROS, Reactive oxygen species; RNS, Reactive nitrogen species; NAC, N-acetylcysteine; CM-H₂DCFDA (or CM), 5-(and 6-) Chloromethyl-2,7-dichlorodihydrofluorescein diacetate; DAF, 4-Amino-5methylamino-2',7'-difluorescein diacetate; MS, Mitosox; GCR, Galactic cosmic rays; LET, Linear energy transfer; HDR, High dose rate; LDR, Low dose rate

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the opposing effect [12]. Similarly, hydrogen peroxide is toxic to neural cell types in organotypic slices [13] and in vivo [14,15], whereas superoxide has been found to be neuroprotective to all main cellular lineages in the CNS [12,16]. The foregoing highlights the sensitivity of the CNS to changes in redox state, and underscores how alterations in the balance between pro- and antioxidants in the brain can have long lasting consequences.

While specific brain cells (i.e. multipotent precursors) exhibit marked sensitivity to irradiation [17,19] the mature and post-mitotic cells comprising the bulk of the CNS are relatively radio-resistant [18,20]. Adverse events in the CNS associated with late normal tissue injury typically occur at prolonged post-irradiation times and generally manifest after relatively higher clinical doses (≥ 40 Gy) [18,20]. At lower doses (1–10 Gy) however, stem cell depletion transpires in neurogenic areas, leading to impaired neurogenesis and synaptic and neuronal remodeling with multifaceted consequences on cognitive function [19,20]. Less clear however, are how these same cell systems respond to even lower doses of sparsely ionizing charged particles and photons, and if/how altered dosing paradigms might impact redox homeostasis and neural stem cell function.

To address the foregoing, we have expanded our past research and focused on the consequences of low dose and dose rate exposure of protons and photons on neural stem cells. Here we report our findings detailing and comparing the temporal response of radiation-induced oxidative stress in rodent and human neural stem cells, and how alterations in irradiation schedule impacts adaptive changes that alter survival and redox equilibrium.

Materials and methods

Cell culture

Multipotent neurosphere cultures derived from wild-type C57BL/6 mice were maintained in suspension, in untreated T-25 flasks under standard conditions which included passaging in serum-free DMEM/F12 supplemented with FGF (20 ng/ml, Peprotech, Rocky Hill, NJ) and EGF (250 ng/ml, Biomedical Technologies Inc., Stoughton, MA) growth factors. ENStem-A human neural stem cells (Millipore, Billerica, MA) were maintained as previously described [22] and their use was approved by the UC Irvine Human Stem Cell Research Oversight Committee. Briefly, cells were passaged on poly-L-ornithine (PLO, 20 μ g/ml, Sigma-Aldrich, St. Louis, MO) and laminin (5 μ g/ml, Sigma-Aldrich) coated flasks in ENStem-A neural expansion media (Millipore) containing neurobasal media supplemented with L-glutamine (2 mM, Invitrogen, Carlsbad, CA), basic fibroblast growth factor (20 ng/ml), B27 and leukemia inhibitory factor (Millipore). For experiments utilizing the antioxidant N-acetylcysteine (NAC, Sigma-Aldrich), NAC was prepared in media the day of use.

Irradiation conditions

24 h prior to irradiation, mouse neurosphere cultures were passaged 1:4 into T-25 flasks for oxidative stress studies or seeded at 10 or 25 k cells per well of a 24 well low-binding tissue culture plate. Human neural stem cells were prepared in a similar fashion with seeding at 50 k cells per well of a 24 well PLO and laminin coated tissue culture plate. Proton irradiations were performed using the Loma Linda University Proton Research Facility synchrotron accelerator using 250 MeV plateau phase protons. Low dose rate (LDR) protons were delivered at 20–25 cGy/h whereas high dose rate (HDR) protons were delivered at 25–50 cGy/min.

For γ -irradiation, a ^{137}Cs irradiator (J.L. Sheppard and Associates Mark I, CA, USA) was used at a dose rate of 2.2 Gy/min.

Oxidative stress

Fluorogenic dyes were used to assess ROS/RNS, nitric oxide and superoxide levels in the mouse neurospheres and human neural stems at various time points following irradiation. Exponentially growing cells were treated for 1 h at 37 °C prior to flow cytometry with 5-(and 6-) chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, 5 μ M, Invitrogen) for ROS/RNS detection. The CM-H₂DCFDA is a cell permeable dye that upon hydrolysis and oxidation yields a green fluorescent signal that can be quantified. Similarly, cells were treated with 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF, 5 μ M, Invitrogen) for nitric oxide detection. The reaction of NO with DAF leads to the production of a nitrosative product that yields a fluorescent signal that can be quantified upon excitation. Lastly, cells were treated with MitoSox (0.5 μ M, Invitrogen) for superoxide detection. Oxidation of this dye by superoxide (and other oxidants) yields an ethidium derivative that exhibits a red fluorescent signal for quantification. Following dye loading, cells were then harvested and assayed using the EasyCyte flow cytometer (Millipore) and the data were analyzed using FCS Express (De Novo Software, Los Angeles, CA). For antioxidant measurements, NAC was added directly after irradiation and replenished with a media change 24 h later. The data are averaged from at least three determinations and normalized to the unirradiated controls for each time point and dye.

Cell survival analysis

As previously described, mouse neurospheres or human neural stem cells seeded in 24 well plates were frozen without media at -80 °C overnight 5 days after the last irradiation dose, then lysed with Mammalian Protein Extraction Reagent (Thermo Fischer Scientific, Waltham, MA) containing $2.5 \times$ SYBR Green I (Invitrogen). SYBR Green I fluorescence was detected using a SynergyMX microplate reader (BioTek, Winooski, VT) using the following settings, excitation 497 nm, emission 520 nm. Cell counts were determined using applicable standard curves previously generated for each cell type. For antioxidant measurements, NAC was added directly after priming dose irradiation, and removed 2 h prior to challenge dose irradiation. Following subsequent irradiation NAC was replenished at the indicated concentration. The data are averaged from at least three determinations and normalized to the unirradiated controls.

Data analysis and statistics

The flow cytometry data for ROS/RNS, nitric oxide and superoxide levels are presented normalized to the unirradiated controls for each time point per assay dye. For the cell survival assays, the data are normalized to the unirradiated controls. The data were assessed for significance ($P=0.05$) by analysis of variance (ANOVA) with comparisons between groups performed using Newman-Keuls post-hoc tests.

Results

Dose rate effects of proton irradiation on oxidative stress levels

We have previously demonstrated that proton irradiation of rat neural precursor cells elicited a rapid rise in ROS following doses between 1 and 10 Gy [6]. Here we investigated the effect of

low dose proton irradiation. Mouse neurosphere cultures were exposed to proton irradiation at LDR and HDR at doses between 10 and 50 cGy to examine changes in ROS/RNS and nitric oxide levels. The resulting changes in ROS/RNS were temporally different between the LDR and HDR irradiations. Overall, neurospheres receiving LDR irradiation showed decreased levels of ROS/RNS with significant changes at all time points receiving 50 cGy (Fig. 1A). The lowest doses of 10 and 30 cGy also elicited smaller perturbations in the ROS/RNS levels over the 48 h time course, with significant decreases achieved at 36 h for 10 cGy, in addition to 36 and 48 h for the 30 cGy dose (Fig. 1A). Neurospheres receiving HDR irradiation demonstrated a cyclic response in ROS/RNS levels. Significant increases were observed at 6, 12 and 36 h post-irradiation for the 50 cGy dose, interspersed with significant decreases at 24 and 48 h (Fig. 1B). The later time points also demonstrated significant decreases for the 10 and 30 cGy doses with a return to baseline at 36 h for the 10 cGy dose and a near return to baseline for the 30 cGy dose; this dose remained statistically decreased relative to controls with a similar result also seen at 6 h (Fig. 1B).

Temporal changes in nitric oxide levels did not track those seen for ROS/RNS following low dose proton irradiation of neurospheres. Responses were small overall for both dose rates. For the LDR irradiations, nitric oxide levels were generally depressed or at baseline over the 48 h post-irradiation interval, with significant decreases seen at 6 and 48 h for the 30 cGy dose as well as 48 h for the 50 cGy dose (Fig. 1C). Interestingly, all doses produced an increase in nitric oxide levels at 36 h with significance achieved for the 30 and 50 cGy doses. The response to HDR irradiation was also somewhat muted

with respect to changes in nitric oxide but differed from the LDR temporal evolution. At 6 h, HDR doses increased nitric oxide levels with significant changes detected for the 30 and 50 cGy doses (Fig. 1D). Nitric oxide levels then continued to decrease with a minimum level achieved at 36 h where significant decreases were observed for the 10 and 50 cGy doses (Fig. 1D). At 48 h, nitric oxide levels were increased relative to 36 h and significantly increased for the 30 cGy dose, although the 10 cGy remained significantly decreased compared to unirradiated controls (Fig. 1D).

Human neural stem cell oxidative stress levels are altered by proton irradiation

We examined the effect of low dose and dose rate proton irradiation on human neural stem cells and found that changes in ROS/RNS levels were quite significant. At the 10, 25 and 50 cGy doses delivered at 20–25 cGy/h, ROS/RNS levels increased significantly by 36%, 56% and 37% respectively 6 h after irradiation (Fig. 2A). A similar increase was not seen in cells receiving the high dose rate (25–50 cGy/min) where ROS/RNS levels were below controls at 6 h post-irradiation and remained depressed until 36 h (Fig. 2B). At 36 h post-irradiation both dose rates resulted in significant increases in ROS/RNS levels. For LDR, 10, 25, 50 and 100 cGy doses increased ROS/RNS levels by 43%, 57%, 121%, and 101% respectively, whereas for the same doses at HDR, ROS/RNS levels were increased further by 149%, 134%, 119% and 183% respectively (Fig. 2A and B). These increases were either sustained or elevated further by 48 h, with levels at 100% to 300% above controls for both dose rates, with the largest increase (405%) found for the 10 cGy dose at HDR (Fig. 2A and B).

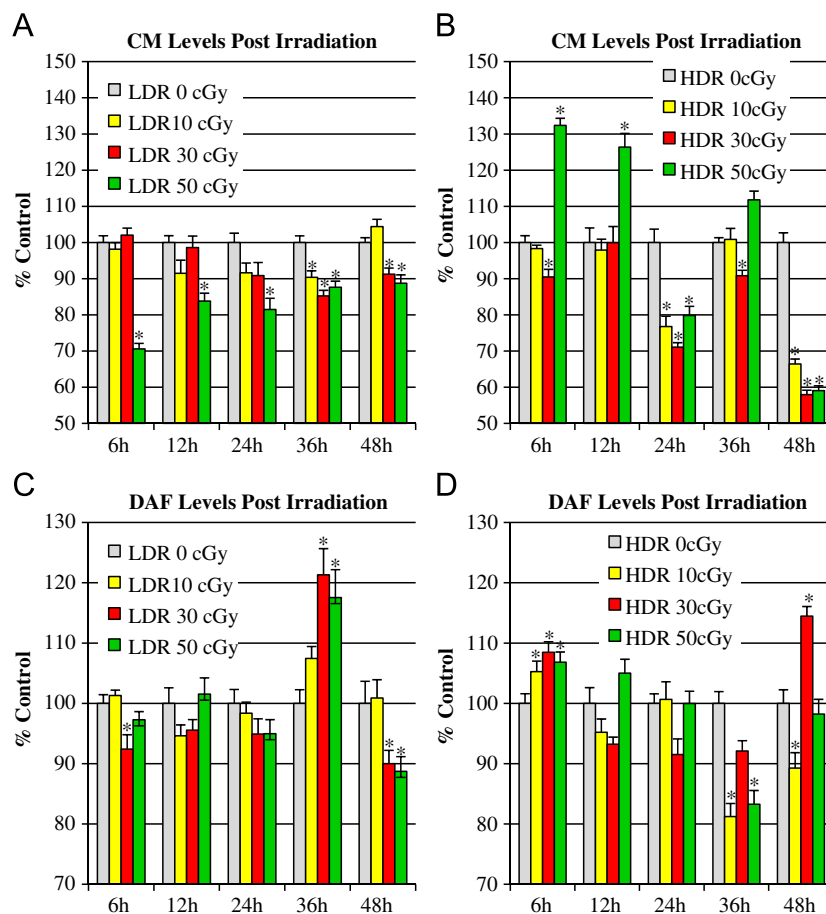


Fig. 1. Mouse neural precursor cells exposed to 250 MeV protons exhibit perturbations in ROS/RNS and nitric oxide levels. Cells subjected to irradiation were incubated at 37 °C with 5 μ M CM-H₂DCEA or DAF-FM for 1 h, harvested and subjected to flow cytometric analysis. One way ANOVA followed by Newman–Keuls test to compare 0 Gy group with corresponding irradiated samples. * $P < 0.05$.

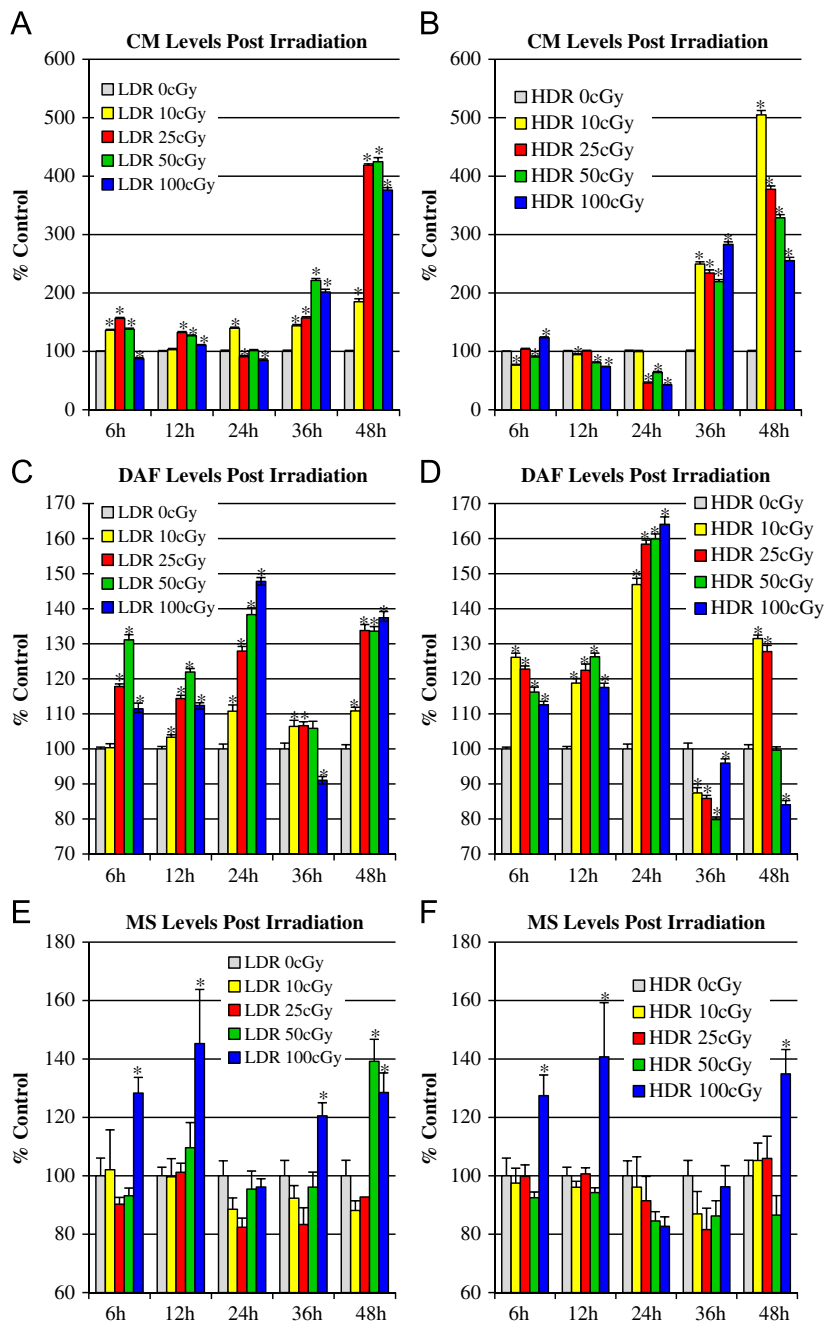


Fig. 2. Human neural precursor cells exposed to 250 MeV protons exhibit perturbations in ROS/RNS, nitric oxide and superoxide levels. Cells subjected to irradiation were incubated at 37 °C with 5 μ M CM-H₂DCFA, DAF-FM or MitoSox for 1 h, harvested and subjected to flow cytometric analysis. One way ANOVA followed by Newman-Keuls test to compare 0 Gy group with corresponding irradiated samples. * $P < 0.05$.

Nitric oxide levels were also found to increase after most proton irradiations (Fig. 2C). At 6 and 12 h post-irradiation, both dose rates gave qualitatively similar rises in nitric oxide levels, with peak levels observed at 24 h. At this time, 10 cGy at LDR increased nitric oxide levels by 10%, while the remaining LDR doses elevated nitric oxide from 30% to 50% (Fig. 2C). At 24 h, the HDR response revealed larger increases in nitric oxide, with levels elevated by 40%–60% above control for all doses (Fig. 2D). Interestingly, at 36 h nitric oxide levels decreased to just above (less than 10%) or even below (at most 20%) control levels but rebounded for all LDR doses by 48 h, along with the 10 and 25 cGy doses at HDR (Fig. 2D).

Analyses of superoxide levels showed smaller fluctuations and were for the most part not significantly elevated (< 10%) or decreased (< 20%) compared to controls for both dose rates (Fig. 2E

and F). Superoxide levels were however, significantly elevated at the higher doses used in these experiments. While a dose of 50 cGy at LDR elevated superoxide levels at 48 h by 40%, a 100 cGy dose at LDR was found to increase superoxide levels by 20%–40% at 6, 12, 36 and 48 h (Fig. 2E). A 100 cGy dose at HDR significantly increased superoxide levels by 20%–40% at 6, 12 and 48 h (Fig. 2F).

Modulation of radiation-induced oxidative stress with *N*-acetylcysteine

To establish the capability of an antioxidant to modulate radiation-induced oxidative stress in human neural stem cells, cells were irradiated as detailed above, and then cultured for 48 h in the presence of NAC. Compared to measurements in the absence of the

antioxidant (Fig. 2), NAC was able to reduce the level of reactive species present 2 days following exposure to low dose gamma irradiation (Fig. 3). Significant reductions in the level of ROS/RNS were found after NAC treatments for each dose analyzed (Fig. 3A). Radiation-induced nitric oxide (Fig. 3B) and superoxide (Fig. 3C) levels were also attenuated in the presence of NAC, although under most situations reduced levels of nitric oxide and superoxide were not significant due to the relatively small increases in these species found after irradiation of untreated cells. While NAC was found to reduce radiation-induced oxidative stress, further reduction of reactive species to background levels or below was not achieved at all the doses (Fig. 3).

Low dose priming Improves neural stem cell survival

To investigate whether low dose and/or low dose rate priming would yield improved survival after a higher challenge dose, we irradiated rodent neurospheres with either 30 cGy of 250 MeV protons at the low and high dose rates already described. One day

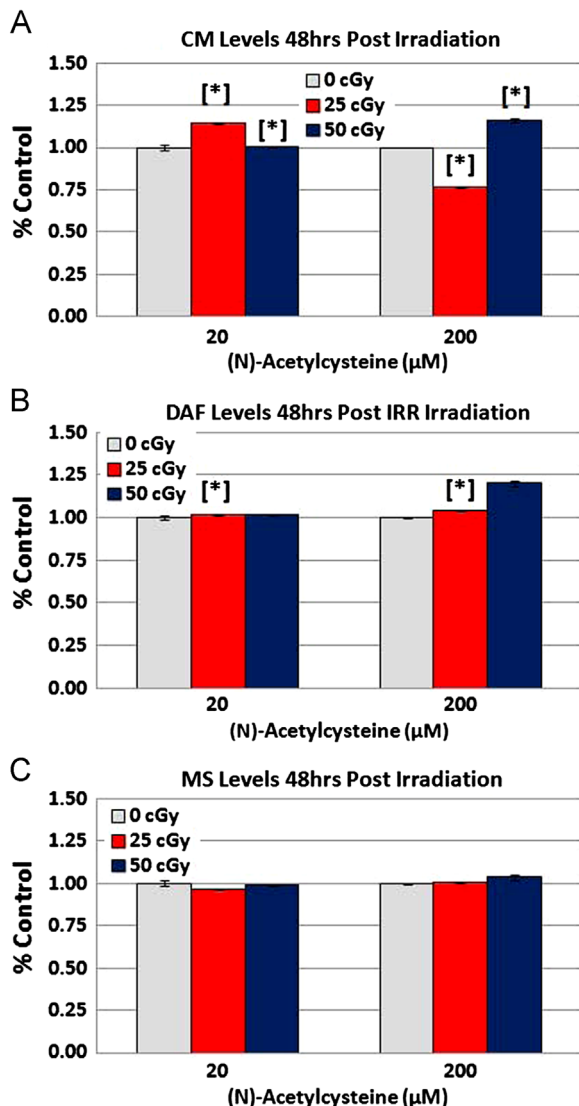


Fig. 3. Antioxidant treatment reduces radiation-induced oxidative stress in human neural stem cells. Treatment with the antioxidant N-acetylcysteine (NAC) immediately following irradiation suppressed ROS/RNS (A) and nitric oxide levels (B) significantly, while superoxide levels were found unchanged (C) when measured 48 h after exposure. One way ANOVA with unpaired *t*-test. * $P < 0.0001$ in comparison with CM and DAF levels at HDR 25 and 50cGy (see Fig. 2B, D).

after the priming dose, a larger 5 Gy challenge dose of γ -irradiation was delivered to the neurospheres. The primed and challenged cells were maintained in culture for an additional 5 days post-challenge then harvested for cell quantification. The challenge irradiation significantly decreased survival to 39% compared to unirradiated controls whereas neurospheres receiving the 30 cGy LDR proton priming dose exhibited a significantly improved post-challenge survival of 65% (Fig. 4A). Under similar conditions and comparisons, neurospheres also showed a trend toward improved survival of 50% of controls after HDR proton priming, an effect however, that did not reach statistical significance (Fig. 4A).

We also determined that acute exposure to HDR protons decreased neurosphere survival 5 days post-irradiation. We found that 10 and 30 cGy doses decreased survival to 95% and 85% of unirradiated controls (Fig. 4B). As expected, the larger 200 and 500 cGy single doses produced significant reductions in survival of

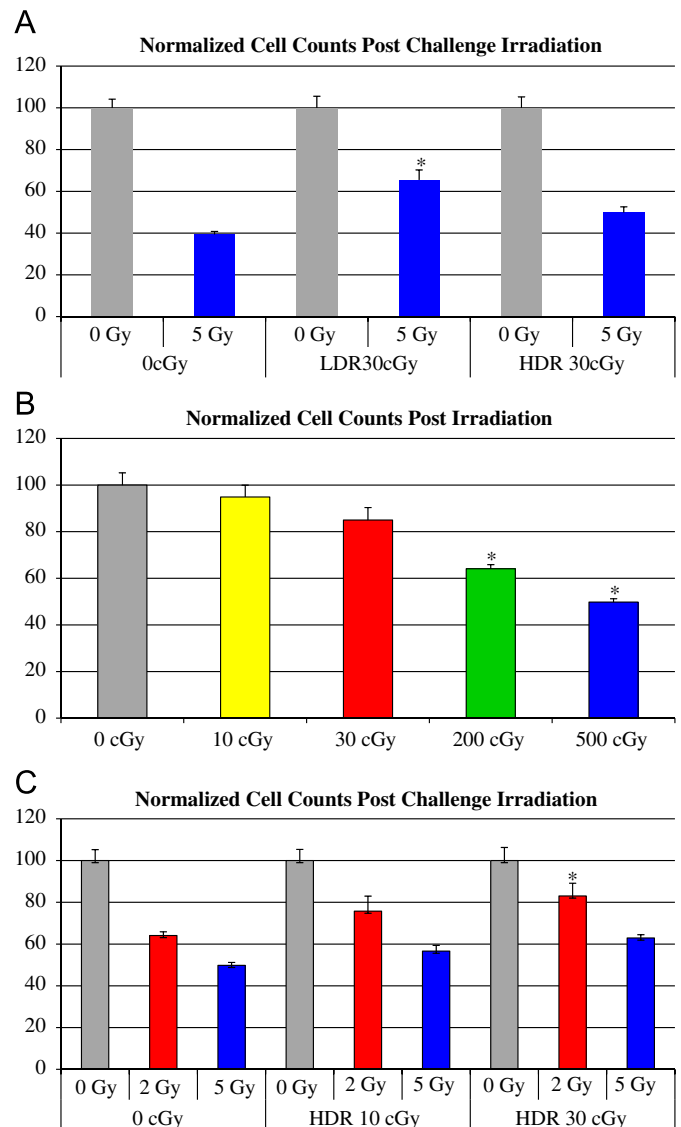


Fig. 4. Mouse neural precursor cells exposed to priming doses to irradiation exhibit improved survival following a larger challenge irradiation. Cells were primed and challenged with 250 MeV protons or γ -rays as follows (A) proton primed, γ -ray challenged (B) Single dose HDR proton (C) HDR proton primed and challenged. Cells were harvested 5 days post-challenge and quantified for cell survival, represented as a percentage of the cells receiving no priming and no challenge dose. One way ANOVA followed by Newman-Keuls test to compare no prime, 5 Gy group (A); no prime, 2 Gy group (C); or 0 Gy group to corresponding irradiated samples (B). * $P < 0.05$.

64% and 50% of unirradiated control (Fig. 4B). To examine further the effects of prior low dose irradiation on the survival of neurospheres, cells were irradiated with 10 and 30 cGy of HDR protons for the priming dose followed by either a 2 or 5 Gy challenge dose of HDR protons 24 h later. While acute doses of 2 or 5 Gy reduced survival as indicated above, both priming doses improved survival after a 2 Gy challenge dose; the 10 cGy priming dose improved survival to 76% of controls and the 30 cGy priming dose significantly improved survival to 83% of controls (Fig. 4C). Similarly, both priming doses displayed a trend towards improved survival following the 5 Gy challenge dose; 10 and 30 cGy led to survival of 57% and 63%, respectively, neither of which reached statistical significance above the non-primed neurospheres (Fig. 4C).

We further investigated whether low dose and dose rate priming improved survival in the human neural stem cells. Similar to the rodent neurospheres, hNSCs were primed with both LDR and HDR 250 MeV protons. The hNSCs were given 25 cGy prior to 1 and

2 Gy γ -irradiation challenge doses. Two trends were immediately noticeable. Both priming dose rates significantly improved survival following the 1 Gy challenge dose. Survival of 85% and 90% was found for the LDR and HDR respectively, compared to 68% for the non-primed cells (Fig. 5A). Despite this finding, neither priming dose was found to be beneficial for survival after a 2 Gy challenge dose. Survival of 34% and 20% was found for the LDR and HDR primed cells respectively, compared to 51% for the non-primed cells (Fig. 5A). When hNSCs were treated with both proton priming and challenge (HDR) doses similar trends were seen with the 1 and 2 Gy challenge doses. The 25 cGy priming showed a trend of improved survival following the 1 Gy challenge, but only after LDR (Fig. 5B). As before, neither priming dose improved survival after the 2 Gy challenge. Lastly, we examined whether survival was improved after a priming dose of γ -rays followed by 1 Gy challenge of HDR protons. Similar to prior results, we found that γ -ray priming doses were sufficient to improve hNSC survival significantly (68%), compared to non-primed cells (46%, Fig. 5C).

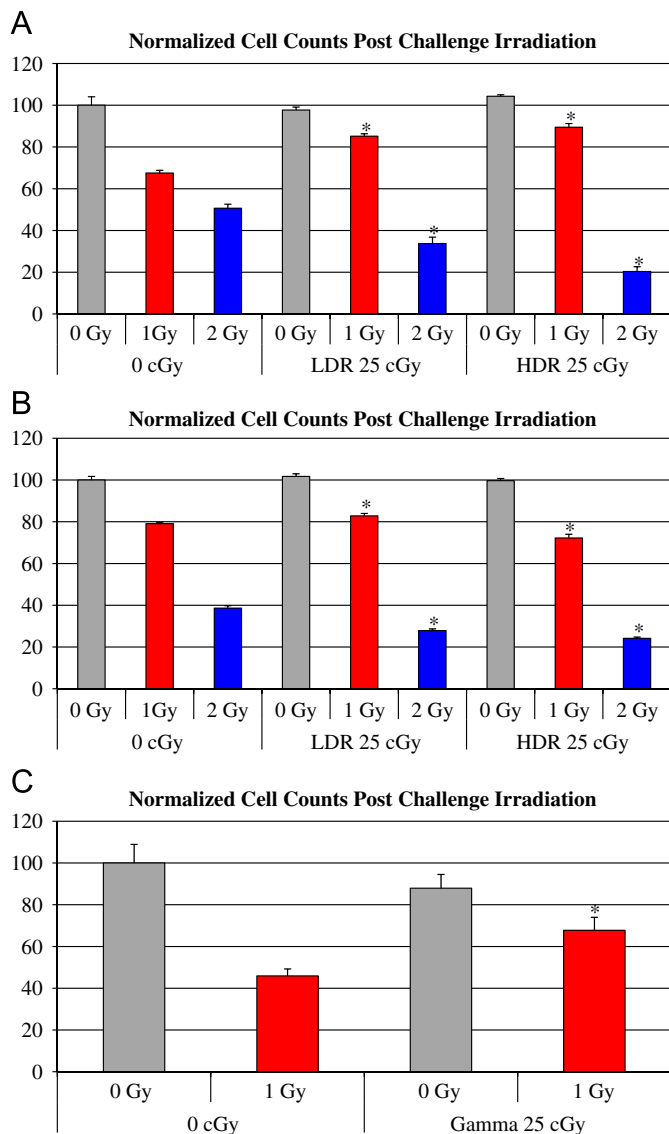


Fig. 5. Human neural precursor cells exposed to priming doses to irradiation exhibit improved survival following a larger challenge irradiation. Cells were primed and challenged with 250 MeV protons or γ -rays as follows (A) proton primed, γ -ray challenged (B) proton primed and challenged and (C) γ -ray primed, proton challenged. Cells were harvested 5 days post-challenge and quantified for cell survival, represented as a percentage of the cells receiving no priming and no challenge dose. One way ANOVA followed by Newman-Keuls test to compare no prime, 1 and 2 Gy groups (A, B); or no prime 1 Gy group (C). * $P < 0.05$.

Antioxidant modification of human neural stem cell survival following low dose priming

The capability of low dose irradiation to elicit oxidative stress suggested that the pro-oxidant state of the neural stem cells might underlie the improvements in survival found after priming doses. To investigate this possibility, human neural stem cells were treated with NAC directly after irradiation to determine if reducing the levels of reactive species could attenuate subsequent survival. In the absence of the priming dose, irradiation (1 Gy) reduced survival as expected, which was slightly higher (although not significantly) when NAC was added afterwards (Fig. 6). Cells subjected to low dose priming (25 cGy) in the absence of NAC had significantly improved survival following the higher challenge dose (1 Gy, Fig. 6). This finding corroborated similar measurements (Fig. 5c), where priming dose exposure again led to an increase in survival (~20%) compared to unprimed cells (Fig. 6). Interestingly, treatment with NAC was found to eliminate the benefit of the priming dose on survival when compared to untreated cells (Fig. 6). Thus, while NAC was not present during either irradiation, its presence was sufficient to eradicate any impact of the priming dose on survival.

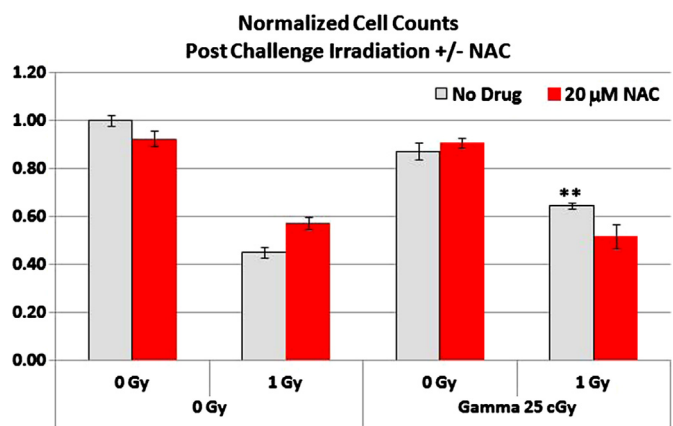


Fig. 6. Antioxidant treatment eliminates the beneficial effects of priming doses on human neural stem cells survival. Cells were irradiated and challenged with 1 Gy (γ -ray) 24 h following a 25 cGy (γ -ray) priming dose in the absence or presence of NAC added directly after (but not present during) irradiation. Cells were harvested 5 days post-challenge and quantified for cell survival, represented as a percentage of the cells receiving no priming and no challenge dose. One way ANOVA with unpaired *t*-test. ** $P < 0.002$ compared to 1 Gy (no priming).

Priming alters hNSC oxidative stress levels following challenge doses

To examine the relationship between oxidative stress and survival in hNSC subjected to proton priming (25 cGy) and γ -ray challenge (1 Gy) doses, we analyzed cells via flow cytometry for ROS/RNS, nitric oxide and superoxide levels at various time points after the challenge dose. Particularly evident from the analysis of primed hNSCs was that radiation-induced ROS/RNS levels were increased regardless of the dose rate (Fig. 7A). For example, between the 6 and 48 h time points, LDR 25 cGy priming (no challenge) increased ROS/RNS levels to between 43% and 267% above controls, with peak levels occurring at 36 h post-challenge (Fig. 7A). Similarly, for the 25 cGy HDR priming (no challenge), ROS/RNS levels were increased to 44% and 477% between 6 and 48 h also with peak levels also at 36 h (Fig. 7A).

Given the effect of the priming doses alone, we examined further the changes due to the challenge doses compared to its own priming group. This analysis revealed that the priming doses tended to blunt the subsequent ROS/RNS response of cells to the 1 Gy challenge dose (i.e. doses were not additive). For example, for the non-primed cells, 1 Gy alone elevated ROS/RNS to 28%, 19%, 32%, 77% and 120% at 6, 12, 24, 36 and 48 h post-challenge. However, for cells primed at 25 cGy LDR, these levels were 17%, 3%, 66%, -78% and 85% at the same time points and 7%, 28%, -14%, 24%, and -41% for cells primed at 25 cGy HDR (Fig. 7B).

There were similar changes seen with the acute 2 Gy dose in terms of increased ROS/RNS levels over unirradiated controls. In this situation however, the priming doses were somewhat less effective at suppressing the ROS/RNS response of cells to the higher challenge dose. For example, comparing the ROS/RNS levels of cells primed at 25 cGy LDR, showed that a 1 Gy dose led to changes of 17%, 3%, 66%, -78% and 85% while a 2 Gy dose led to changes of 13%, 44%, 64%, 158%, and 141% at 6, 12, 24, 36 and 48 h post-challenge, as compared to cells just given the priming dose (Fig. 7B).

Trends were less apparent for radiation-induced nitric oxide levels. In general the 1 Gy challenge caused fluctuations between -20% and 11% of unirradiated controls over the 48 h time course whereas the 2 Gy challenge increased levels to 15%–30% above controls, with the peak levels occurring at 6 and 48 h (Fig. 8A). Over this same timeframe, cyclical changes in nitric oxide (increase, decrease then latter increases) were seen in all of the primed groups except for the LDR 25 cGy non-challenged group, which showed small increases up to a maximum level at 36 h followed by a relative decline at 48 h (Fig. 8A). For the 1 Gy challenge, the fluctuations in nitric oxide levels were greater in magnitude for the cells that received priming doses, with increases from 49 to over 80% compared to a maximum of 11% in the non-primed cells (Fig. 8A).

With respect to change in nitric oxide levels above non-challenged controls for each priming group, the LDR and HDR

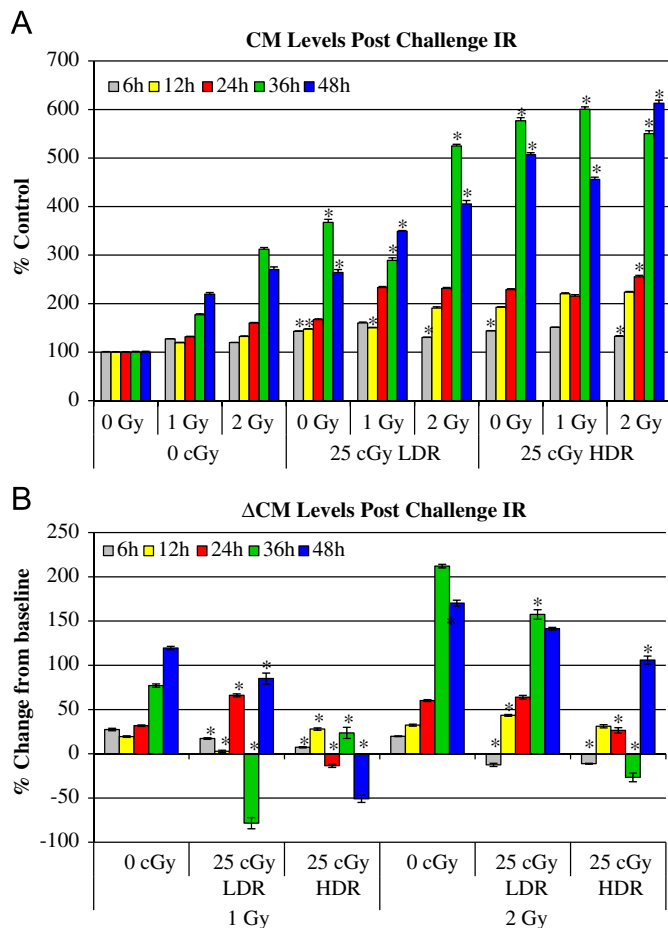


Fig. 7. Human neural stem cells exposed to proton priming and γ -ray challenge doses exhibit increases in CM (ROS/RNS) levels after challenge dose following the (A) no priming, low and high dose rate proton priming doses. Relative changes in ROS/RNS from the 0 Gy baseline challenge dose are shown for the (B) no priming, low and high dose rate proton priming doses. One way ANOVA followed by Newman–Keuls’ test to compare with corresponding no prime groups. * $P < 0.05$.

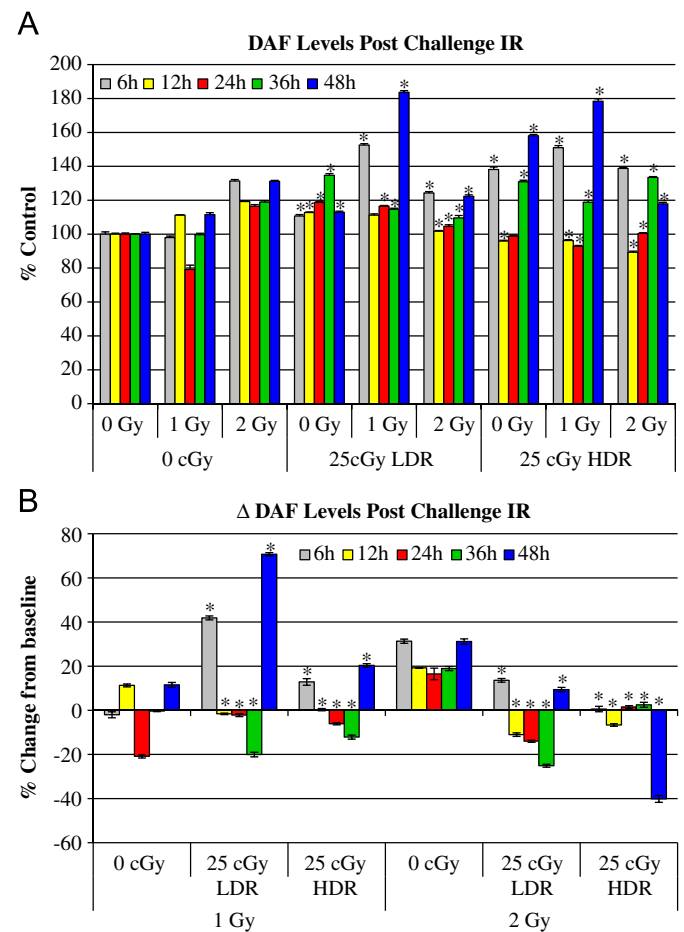


Fig. 8. Human neural stem cells exposed to proton priming and γ -ray challenge doses exhibit increases in DAF (nitric oxide) levels after challenge dose following the (A) no priming, low and high dose rate proton priming doses. Relative changes in nitric oxide from the 0 Gy baseline challenge dose are shown for the (B) no priming, low and high dose rate proton priming doses. One way ANOVA followed by Newman–Keuls’ test to compare with corresponding no prime groups. * $P < 0.05$.

primed cells had larger 6 and 48 h increases compared to the non-primed group for the 1 Gy challenged cells and were relatively decreased at 36 h (Fig. 8B). For the 2 Gy challenged cells, the primed cells in general showed less change above non-challenged cells within each priming group (Fig. 8B).

Under the stated priming/challenge dose conditions, changes in superoxide levels provided the best evidence for radioadaptive effects. In general, for all non-challenged groups, the cells receiving priming did not differ greatly from unirradiated controls, except for 25 cGy LDR at 24 and 36 h as well as 25 cGy HDR at 36 h. Furthermore, overall superoxide levels appeared to be decreased in both priming groups compared to unprimed cells given 1 Gy. Unprimed cells irradiated with 1 Gy, showed increased superoxide levels between 20% and 89% from 6 to 48 h, with peak levels occurring at 36 h (Fig. 9A). For those cells primed at LDR before the 1 Gy challenge, superoxide levels fluctuated between 10% and 52% with peak levels also at 36 h, whereas for cells primed at HDR, superoxide levels varied between 11% and 36% with the peak level found at 48 h (Fig. 9A).

For those cells given 2 Gy, priming seemed to have a smaller impact. The maximum increase of 79% found at 36 h in unprimed

cells, was exceeded twice by the 25 cGy LDR primed cells at 24 and 48 h with levels at 84% and 90% respectively, and once by the 25 cGy HDR primed cells at 36 h with a level of 82% (Fig. 9A). As before, changes in superoxide levels compared to non-challenged controls for each priming group were examined and showed that priming in general, blunted the superoxide response of cells following the 1 Gy challenge. This was especially pronounced at 36 h and to a lesser degree at 48 h, where superoxide levels were all significantly less than non-primed cells, with the exception of the 25 LDR 48 h group, which did not achieve statistical significance (Fig. 9B). The dampening effects of priming on Mitosox levels was less for the 2 Gy challenge groups, as highlighted by the response of LDR primed cells at 24 and 48 h that significantly exceeded their non-primed counterparts, with a similar effect seen for the HDR primed cells at 24 and 36 h (Fig. 9B).

Discussion

We have undertaken an extensive analysis of the low dose and dose rate response of rodent and human stem cells exposed to sparsely ionizing radiation. Data revealed a complex temporal evolution of radiation-induced reactive species, that was generally, but not always dose-responsive. Past work from us and others have found qualitatively similar responses, where low LET irradiation elicits acute and persisting changes in the redox profile of surviving cells [6–8,21]. For rodent cells exposed to doses up to 50 cGy, ROS/RNS levels detected after LDR showed little change, but exhibited cyclical variations with dose after HDR exposures (Fig. 1A and B). These patterns were different for nitric oxide levels that showed considerable variability between the doses and dose rates used (Fig. 1C and D). For each of the dyes, larger variations were noted for HDR irradiations, and with a few exceptions, LDR exposures elicited lower levels of reactive species over 2 days in rodent cells.

For human neural stem cells exposed acutely to proton doses ranging from 10 to 100 cGy, radiation-induced changes in oxidative stress were more pronounced. Reactive species detected by the CM dye were markedly elevated after 24 h, and exhibited qualitatively similar responses to either dose rate, with the largest increases found after HDR (Fig. 2 A and B). Nitric oxide levels were found to increase at earlier times and over most dosing paradigms, with relative maxima occurring 24 h after exposure (Fig. 2C and D). Both dose rates again gave qualitatively similar responses, with more robust increases found after HDR. Superoxide levels in hNSCs were least responsive to acute proton irradiation, and were generally depressed below control values except at the higher doses (Fig. 2E and F). While general trends were difficult to discern, it was clear that neural stem cells exposed acutely to protons over the range of 10–100 cGy exhibited marked fluctuations in ROS and RNS levels over the subsequent 2 days. The hNSCs were more sensitive to radiation-induced changes in oxidative stress than their rodent counterparts, and showed higher levels of reactive species when doses were constrained to shorter intervals. The capability to modulate radiation-induced oxidative stress using NAC confirmed the expected activity of this agent in cells (Fig. 3A–C). More importantly however, use of NAC provided the means to test whether radiation-induced reactive species played a role in the beneficial adaptive effects found in cells subjected to priming and challenge doses (discussed below). The foregoing findings corroborate much of our past work at higher doses [22], and now confirm the capability of lower doses and dose rates to elicit significant changes in radiation-induced oxidative stress.

As space travel will entail exposure to multiple ionizing species at various dose rates, we evaluated the response of neural stem

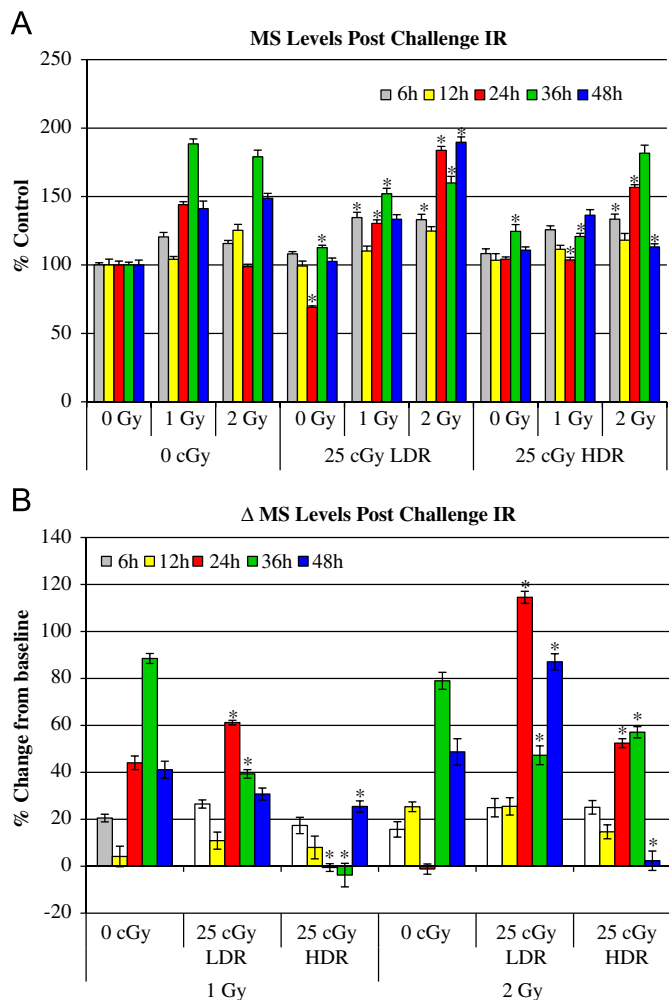


Fig. 9. Human neural stem cells exposed to proton priming and γ -ray challenge doses exhibit increases in Mitosox (superoxide) levels after challenge dose following the (A) no priming, low and high dose rate proton priming doses. Relative changes in superoxide from the 0 Gy baseline challenge dose are shown for the (B) no priming, low and high dose rate proton priming doses. One way ANOVA followed by Newman-Keuls' test to compare with corresponding no prime groups. * $P < 0.05$.

cells exposed to various combinations of protons and gamma rays delivered at LDR and HDR. Other groups have found that under similar irradiation paradigms low dose exposure can alter cytokine expression profiles [23], reduce circulating leukocytes [24] as well as attenuate neoplastic transformation [25]. In the present study, neurospheres primed with low doses of protons and challenged with higher doses of γ -rays or protons showed evidence of improved survival. Compared to cells irradiated acutely with 5 Gy, a LDR priming dose of 30 cGy of protons improved survival by 26% after a 5 Gy challenge dose of γ -rays (Fig. 4A). When a HDR priming dose was used in a similar dosing scheme survival improved by only 10%, indicating that protracting the priming dose had a more beneficial impact on survival for cells subjected to secondary higher dose exposures. For cells subjected to multiple HDR proton exposures, similar radioadaptive effects were found. Compared to acutely exposed cells, priming doses of 10 or 30 cGy improved neurosphere survival by 12% and 19% respectively, following a 2 Gy challenge (Fig. 4C). Under a similar dosing scenario, protective effects were less evident, as 10 or 30 cGy priming doses improved survival after a 5 Gy challenge by 7% and 13% respectively (Fig. 4C). Compared to hNSCs, neurospheres were relatively resistant to the effects of acute, HDR proton exposures, as 5 Gy doses reduced cell numbers by 50%. Reduced sensitivity is not likely a result of hypoxia at the time of irradiation, as neurospheres passed routinely 1-day prior contained $\sim 280 \pm 45$ cells with an average radius of $36 \pm 2 \mu\text{M}$ [20]. The reduced sensitivity of neurospheres may however, reflect inherent differences between 3- and 2-dimensional growth states. Higher order interactions within cellular aggregates such as neurospheres, may approximate 3-D tissue interactions in vivo, conditions that have been found to be protective in other cell and animal models of the CNS [26].

Analogous experiments conducted on hNSCs revealed qualitatively similar findings as those obtained with the rodent cells. In general, dose rate effects were less evident in hNSCs, and radioadaptive changes were only evident at the lower challenge dose of 1 Gy, likely due to their enhanced sensitivity to irradiation. Compared to hNSCs exposed acutely to γ -rays, priming doses of 25 cGy of protons at LDR and HDR improved survival by 18% and 22% respectively, following a 1 Gy challenge of γ -rays (Fig. 5A). Under similar dosing paradigms improved survival was not found after the higher 2 Gy challenge dose of γ -rays (Fig. 5A). Reversing the order of ionizing species also yielded equivalent results, where compared to cells exposed acutely to protons, a 25 cGy dose of γ -rays improved survival by 22% following a 1 Gy challenge dose of protons (Fig. 5C). Interestingly, when only protons were used in the dual dosing paradigms described, radioadaptive effects were not found, possibly due to a higher sensitivity to proton irradiations and/or differences in dose deposition patterns between photons and charged particles (Fig. 5B).

Subsequent studies to uncover the basis for the protective radioadaptive effects on survival were focused on human neural stem cells. Treatment of cells with NAC was found to eliminate the protective effects of the priming dose on survival (Fig. 6), suggesting that radiation-induced oxidative stress played a role in initiating and/or maintaining pro-survival signaling that was sensitive to the redox state of cells. The protective radioadaptive effects described above might be expected after low dose exposure, where more subtle effects are not masked by the electron fields generated at higher doses [27]. To further explore how underlying changes in oxidative stress might impact radioadaptation, hNSC were subjected to analogous irradiation schemes (proton priming, γ -ray challenge) and analyzed for total and relative changes in reactive species over the following 2 days. For hNSCs subjected to priming or challenge doses alone, reactive species were generally elevated over a 6–48 h interval post-challenge. This was particularly evident for ROS/RNS mediated oxidation of the CM dye, where acute exposures led to significant

increases in fluorescence that ranged from 1 to nearly 5-fold over unirradiated controls (Fig. 7A). Similar findings were apparent for superoxide levels, but lower in magnitude and significant only after the higher challenge doses (Fig. 9A). Increased nitric oxide levels from acute priming or challenge doses were relatively modest in comparison (Fig. 8A).

In hNSCs a comparison of relative changes in radiation-induced reactive species revealed more intriguing trends however, and suggested that radioadaptive changes might depend on the suppression of ROS/RNS levels after irradiation. Evidence in support of this comes from the analysis of cells treated with the CM and Mitosox dyes. Relative changes in ROS/RNS levels were suppressed by both LDR and HDR proton priming, but more significantly after the lower 1 Gy challenge dose (Fig. 7B). The more efficient suppression of reactive species under these conditions may explain why hNSCs exhibit improved survival after 1 but not 2 Gy challenge doses of γ -rays. Further evidence supporting this possibility is evident from the relative changes found for radiation-induced superoxide levels. Overall superoxide levels were suppressed in both groups of primed cells compared to unprimed cells given 1 Gy, an effect that was again less evident at the higher challenge dose of 2 Gy (Fig. 9B). While trends were less convincing for radiation-induced nitric oxide levels (Fig. 8B), changes in superoxide levels paralleled changes in survival most closely, suggesting that changes in these species may engage and/or reflect radioadaptive changes in signaling that can impact survival. Past work in non-irradiated neural progenitor cells, has found that oxidative preconditioning under non-toxic levels of hydrogen peroxide provided significant protection against subsequent exposures to lethal levels of this agent [28]. More recent work using charged particles has defined the importance of dose, radiation quality and oxidative stress for mediating non-targeted effects [29–31], and while so-called “bystander effects” were not the focus of this investigation, many of these and related low dose findings may play a role in modulating the responses reported here [27,32]. For non-challenged cells, priming did not alter superoxide levels compared to unirradiated controls, while attenuating superoxide under the same conditions led to protective radioadaptive effects on survival. Further elucidation of these interactions is clearly necessary to more convincingly define cause and effect, but the data do suggest the importance of radiation-induced oxidative stress for mediating physiologically relevant changes in rodent, and in particular, hNSCs.

While the measurement of radiation-induced oxidative stress through the use of fluorogenic dyes provides a convenient and useful measure for determining how specific treatments impact the redox state of cells, there are a number of limitations that need to be considered, and many of the caveats associated with the use of these dyes has recently been reviewed [33]. It should be emphasized that the intracellular redox chemistry of these dyes is complex, and none of the dyes used react stoichiometrically with any given reactive compound. For example, the hydrolyzed carboxylate anion of CM-H₂DCFDA does not directly react with hydrogen peroxide but is susceptible to one-electron oxidation by the hydroxyl radical ($\cdot\text{OH}$), the reactive intermediate compounds I and II formed via interactions with peroxidases and/or heme proteins, cytochrome c, and other reactive species such as the nitrogen dioxide radical ($\cdot\text{NO}_2$), hypochlorous acid (HOCl) and peroxynitrite (ONOO^-) [33]. Furthermore, artifactual amplification of the fluorescence signal intensity can occur through a redox cycling mechanism involving the DCF radical [33]. Mitosox reacts with mitochondrial superoxide to generate a diagnostic marker product exhibiting red fluorescence (i.e. 2-hydroxymitoethidium, 2-OH-Mito-E⁺), but also reacts with other oxidants such as hydroxyl radical and peroxynitrite to generate alternative species with overlapping fluorescent spectra [33]. Thus, red fluorescence

derived from Mitosox may not entirely be due to mitochondrially-derived superoxide [33]. Reactivity of DAF is not exclusive for NO either, as other NO oxidation products are thought to react with DAF to yield fluorescent adducts [34]. While assigning fluorescent signals derived from oxidized dyes to specific reactive species and precise intracellular locations may not be possible, use of these dyes do provide useful information barring over-interpretation of the data. Future work with new dyes designed to possess more specific reactivity and targeted to intracellular sites of interest will provide additional tools with which to interrogate the types and locations of reactive species generated after irradiation.

Conclusions

Our results shed light on the low dose and dose rate response of neural stem cell systems from rodents and humans. Acute and protracted exposures to the dosing paradigms described trigger temporally complex changes to a wide range of reactive species that persist for days. Increased ROS/RNS were significantly elevated in hNSCs after a range of low dose and dose rate exposures, possibly underlying their relatively higher sensitivity to protons and γ -rays. Under multi-dosing paradigms protective radioadaptive changes were associated with priming-induced suppression of oxidative stress, but were limited in protecting hNSCs to relatively lower (1 Gy) rather than higher (2 Gy) secondary exposures. The capability of the antioxidant NAC to eliminate the beneficial effects of priming doses on survival, suggest that radiation-induced oxidative stress plays a role in the regulation of protective radioadaptive effects in neural stem cells. Our work demonstrates that radiation-induced oxidative stress is a biochemical mechanism capable of impacting stem cell functionality, but also reveals the complexities involved. What is clear at this juncture is that very low doses and dose rates of protons and gamma rays are sufficient to elicit quantifiable changes in oxidative stress that may well be linked to survival. Thus, a more thorough understanding of these effects will facilitate efforts for improving risk estimates associated with radiation exposure in space [5].

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