**Article Full Title:**

Inspired oxygen level and tension modulate the murine skin sparing FLASH effect in electron ultra-high dose rate irradiations

**Short Running Title:**

Oxygen impact on murine FLASH skin sparing

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**Data Availability Statement**

Research data will be shared upon reasonable request to the corresponding author.

**Objective:** This study evaluated the hypothesis that baseline tissue oxygen ($pO_2$) would modulate FLASH damage sparing in murine skin, comparing MeV electron ultra-high dose rate (UHDR) versus conventional dose rate (CDR) irradiations.

**Approach:** Murine leg skin $pO_2$ was systematically varied and measured during irradiation from a Mobetron 9 MeV linac at 25 Gy, comparing UHDR (≈240 Gy/s) to CDR (≈0.16 Gy/s), for skin damage outcomes. Radiolytic oxygen consumption, $g_{O2}$ (mmHg/Gy), was also quantified in vivo. Baseline tissue $pO_2$ was systematically modulated in 5 different treatment cohorts, using known methods of altering the inhaled gas (room air, 100% oxygen, or carbogen) and through applied limb vascular compression (partial or full). Induced skin damage was scored daily per mouse.

**Main Results:** FLASH skin sparing was observed in groups with partial leg clamping ($pO_2$≈7±4mmHg), inhaled air ($pO_2$≈12±6mmHg) and oxygen (tissue $pO_2$≈16±4mmHg), while reduction in ulceration progression was significant just in the air inhalation group. No FLASH effect was observed at zero oxygen, via complete blood flow occlusion ($pO_2$≈0±1mmHg), or when modulated by inhaled carbogen ($pO_2$≈21±7mmHg). In vivo measurements of radiolytic consumption, $g_{O2}$, correlated to initial pO2 when FLASH was present ($pO_2$≈4-16mmHg) and saturated above $pO_2$>16mmHg. Inspired carbogen induced the highest $pO_2$ and maximum damage at 25 Gy. Reductions in dose to 20 and 15 Gy reduced skin damage with carbogen, but did not result in FLASH sparing.

**Significance:** These findings indicate that tissue $pO_2$ directly modulates in vivo FLASH skin tissue sparing, requiring roughly room air or 100% oxygen anesthetic gas levels for this effect to be present. The effect is diminished by the absence of oxygen or from carbogen induced oxygen values. Variations in dose do not appear to alter this observation at carbogen induced $pO_2$ levels.

## Introduction

Oxygen plays a fundamental role in radiation therapy as demonstrated in cell survival and in vivo studies where the absence of oxygen (hypoxia) can dramatically reduce radiation killing efficacy by a factor of ≈2.7, known as the oxygen enhancement ratio (OER)[1]. Oxygen is one of the most reactive molecules in biology, and its presence amplifies radiation effectiveness through complex reactive oxygen species (ROS) that react with nearly all biological molecules, including oxidation of DNA to extend and fix it via peroxyl formation[2]. Studies have shown that oxygen acts as a modulator of the observed FLASH effect[3–7], a phenomenon characterized by reduced normal tissue toxicity under ultra-high dose rate (UHDR) radiation therapy while maintaining tumor control[8]. A key distinction between tumors and healthy tissues lies in their baseline oxygenation, with solid tumors typically being hypoxic (0-10 mmHg) as compared to normal tissues (10-40 mmHg)[9]. This difference may be particularly relevant given that oxygen is one of the most significant modifiers of radiation damage, thought to be primarily through the formation of peroxyl radicals that "fix" radiation-induced damage[1,10]. Perhaps the most compelling aspect of this is that true $pO_2$ values are rarely known for any tissue being irradiated, and yet the OER effect can dramatically increase or decrease the effective damage from any given dose delivery, and alter the tumor to normal tissue damage ratio. In this study, we examined how prospectively measuring and modulating the tissue $pO_2$ might affect the observation of the FLASH effect.

One of the more interesting features of UHDR delivery is that the dose and radiolytic oxygen induced damage timelines are sufficiently faster than the oxygen supply from the capillaries, so that it is possible to directly measure the oxygen consumed *in vivo* from the radiation ROS[11–14]. A series of studies have shown how to quantify radiolytic oxygen consumption under UHDR

conditions, and in solutions the consumption per unit dose of radiation at UHDR is lower than that at conventional dose rates (CDR)[11,15–18]. This suggests that UHDR may induce less ROS than CDR at isodose levels and thereby potentially less damage. However, these observations have only been effective in *in vitro* solutions and CDR measurements of this are not easily possible.

*In vivo* studies have shown stable and repeatable murine skin tissue oxygen consumption rates[12–14], and reach a plateau of ≈ 0.2 - 0.3 mmHg/Gy above 20 mmHg baseline tissue oxygenation[14]. This finding corroborates previous *in vitro* studies[16–20] showing that oxygen consumption g-value ($g_{O2}$) systematically decreases with oxygen tension below 20 mmHg, with a 50% reduction at approximately 8 mmHg, however no change is observed in $g_{O2}$ at higher levels. Even more important than this is the observation that the apparent $g_{O2}$ values are lower in tumor tissue than normal tissues[11], suggesting that there may be a reduction in ROS mediated damage. While these studies establish a clear relationship between tissue oxygenation and radiation-induced oxygen consumption, the correlation between these radiation-chemical patterns and differential tissue response under UHDR conditions remains unexplored.

Given the well-characterized relationship between tissue oxygenation levels and radiation damage, and the interesting capability to measure $g_{O2}$ *in vivo*[14], this study was developed to explore the relationship between the FLASH effect, oxygen, dose and $g_{O2}$ metrics in a radiation skin response study. Part of the outcome of this work was to determine if tissue $pO_2$ and/or $g_{O2}$ are mechanistically linked to the FLASH-RT effect. Additionally, a secondary goal was to assess if $pO_2$ or $g_{O2}$ measurements provide beneficial information for optimizing the FLASH effect and revealing necessary initial conditions in tissue. Given the dominance of oxygen inducing reactive

oxygen species in radiotherapy that induce substantial damage, understanding how oxygen levels can affect the observation of the FLASH effect was motivation for this work.

## Methods

### Animals

This vertebrate animal study protocol was approved by the institutional IACUC committee, and all procedures carried out complied. One-hundred and fifty male Albino B6 mice were purchased (Jackson Laboratories, Cambridge, MA) at 8-10 weeks of age and housed in the institution vivarium. The population was divided into two groups, for either UHDR or conventional dose rate (CDR), with each group further split into five oxygenation cohorts. Each cohort consisted of 10-11 mice. There is a well-known sex differential in radiation response[1,21], which has been documented in the FLASH effect and so for simplicity only male mice were studied here[7].

Fur on the left leg, flank, and surrounding area of each mouse was shaved 3 days prior to irradiation. One hour before irradiation, all mice underwent a 3-minute anesthesia induction with 3% isoflurane. The oxygen probe Oxyphor PdG4 (Oxygen Enterprises, Philadelphia, PA), at 200μM in 0.05 mL in phosphate buffer solution was systemically administered into the retro-orbital space, with the oxygen measurement procedure described further below. Mice were allowed to recover to permit the diffusion of Oxyphor PdG4 into the murine skin as in prior studies[22]. The mice were then re-anesthetized for radiation delivery, using isoflurane at 3% for induction (delivered at 500 ml/min for 3 minutes) and maintained at 1.5% (delivered at 100 ml/min), under three different gas conditions: room air, 100% oxygen, or carbogen, and three different levels of vascular clamping: no clamping, partial clamping and complete clamping. The anesthesia/gas

inhalation timing followed the protocol reported by Tavakkoli et al.[7]. A heating pad was placed underneath each mouse to maintain consistent body temperature.

**Radiation Delivery & Dosimetry**

Irradiations were performed using a UHDR-capable Mobetron electron accelerator (IntraOp Inc., Sunnyvale, CA) designed for research use. The radiation field was shaped by a custom-designed collimator creating a 1.6 cm diameter circular treatment area, positioned on each mouse's left flank. To ensure accurate dosimetry and simultaneous oxygen measurements, the experimental setup incorporated a 3-D printed PETG cone with integrated holders for both an EDGE™ Detector (Sun Nuclear Corp, Melbourne, FL) and oxygen measurements via a fiber-coupled phosphorimeter. This arrangement positioned the measurement devices upstream of the mouse while maintaining field alignment and preventing beam perturbation. To replicate conditions known to induce a FLASH sparing effect, all mice received a single-fraction dose to the left flank skin using a 1.6 cm circular field, under either CDR or UHDR delivery modes[23].

Dosimetric accuracy was ensured through calibration of the EDGE™ Detector against a flashDiamond detector (PTW, Freiburg, Germany), specifically designed for UHDR applications. The calibration was performed under identical geometric and delivery conditions as the animal irradiations, establishing a conversion factor between diode readings and delivered electron fluence. Both detectors have been validated for UHDR dosimetry in previous studies by Rahman et al. and Tessonnier et al.[24,25]. Temporal pulse information was measured and verified using the Beam Current Transformers (BCTs) installed in the head of the Mobetron, with dosimetric measurements included in Table 1.

| Delivery Parameter | UHDR 25 Gy | UHDR 20 Gy | UHDR 15 Gy | CDR |
|---|---|---|---|---|
| SSD (cm) | 37.0 | 37.0 | 37.0 | 46.0 |
| Dose (Gy) | 24.8 ± 0.4 | 20.4 ± 0.1 | 15.6 ± 0.2 | 24.9 ± 0.50<br>19.9 ± 0.03<br>14.9 ± 0.01 |
| PRF (Hz) | 90 | 90 | 90 | 30 |
| $D_M$ (Gy/s) | 248 ± 4.0 | 230 ± 1.5 | 234 ± 2.9 | 0.16 ± 0.01 |
| Pulse Width (μs) | 3.2 | 3.2 | 3.7 | 1.2 |
| No. of Pulses | 10 | 8 | 6 | |
| Dose/Pulse (Gy) | 2.48 ± 0.04 | 2.55 ± 0.01 | 2.61 ± 0.03 | |
| $D_P$ (Gy/s) | $7.76 \times 10^5 \pm 0.12 \times 10^5$ | $7.91 \times 10^5 \pm 0.05 \times 10^5$ | $7.06 \times 10^5 \pm 0.09 \times 10^5$ | |

***Table 1:*** *Beam delivery parameters for UHDR and CDR irradiations. Key characteristics include source-to-surface distance (SSD), pulse repetition frequency (PRF), pulse width, dose per pulse, instantaneous dose rate ($D_P$), mean dose rate ($D_M$), and total dose delivered, with values here reported as delivered with standard deviation values.*

**Skin Damage Assay**

Following radiation delivery, animals were monitored daily to visually score the progression of skin damage, via fixed geometry white light imaging. The primary endpoint of the study was the magnitude of skin damage score at the irradiation site, with a secondary measure of number of days to skin ulceration, consistent with previous UHDR studies investigating skin response[23,26,27]. The skin damage scoring scale was similar to that previously published by Noda et al.[28], shown in Table 2. As stated previously, the event conditional for a Kaplan-Meier curve analysis for this study was skin ulceration across the irradiation area, which corresponds to a skin score of 2 on this guide. This score was chosen because the observation of distinct ulceration onset was determined to be the most objectively binary decision point between graders for onset of damage. Additionally, each mouse was survived and monitored daily until a skin score of 3 was reached, at which point the mouse was sacrificed based upon IACUC guidance. At the conclusion

of the study, 51 days post-irradiation, all remaining mice were sacrificed regardless of skin score. 51 days was chosen to allow for the observation of recovery in low damage presenting mice[29]. Daily reading scores for each mouse were determined with knowledge and consideration of previous daily scores to track damage progression over time, while the mouse groups (UHDR, CDR, oxygen conditions) were blinded from the grader.

| **Mouse Skin Reaction Scoring Scale** | |
|---|---|
| Score | Description |
| 0 | Normal appearance; no visible changes |
| 0.5 | Questionable change; difficult to distinguish from normal |
| 0.75 | Mild abnormality; subtle changes visible |
| 1 | Clear abnormality with erythema (reddening) |
| 1.25 | Pronounced erythema with white flaky skin and/or edema |
| 1.5 | Localized moist desquamation in a very small area with scaly or crusty appearance |
| 1.75 | Multiple small areas of moist desquamation |
| 2 | Extensive area breakdown with moist regions (ulceration) |
| 2.5 | Large confluent areas of moist desquamation with exudate |
| 3 | Severe skin breakdown affecting majority of irradiated area with extensive moist exudate |

***Table 2:*** *Radiation-induced skin damage numerical score scale used on daily photographs.*

**Tissue Oxygenation Modulation**

As detailed in Figure 1A, five oxygenation conditions were used to modulate tissue oxygen levels: (i) inhaled room air with isoflurane anesthesia, with complete vascular compression to the leg above the irradiation site stopping all blood flow, ("Anoxic")

(ii) inhaled room air with isoflurane anesthesia, with partial vascular compression above the irradiation site inducing restricted blood flow, ("Hypoxic to mildly hypoxic)"

(iii) room air with isoflurane anesthesia, with no leg ligation (21% $O_2$), ("Mildly hypoxic")

(iv) inhaled 100% oxygen gas with isoflurane anesthesia, with no leg ligation, ("Normoxic to Mildly hyperoxic)

(v) inhaled carbogen gas, here defined as 95% $O_2$ with 5% $CO_2$, with anesthesia, with no leg ligation. ("Hyperoxic")

These approaches achieved a range of initial $pO_2$ levels from 0 to 43 mmHg in the skin of the mouse leg (Figure 1C). Both the UHDR (n = 54) and CDR (n = 55) groups were divided (n = 10-11) into these five cohorts to evaluate the FLASH effect under varying oxygen conditions, given the expected impact of oxygen levels on radiation response[1]. An additional cohort of UHDR (n = 20) and CDR (n = 21) mice were irradiated at 20 or 15 Gy dose levels under carbogen conditions due to assay saturation.

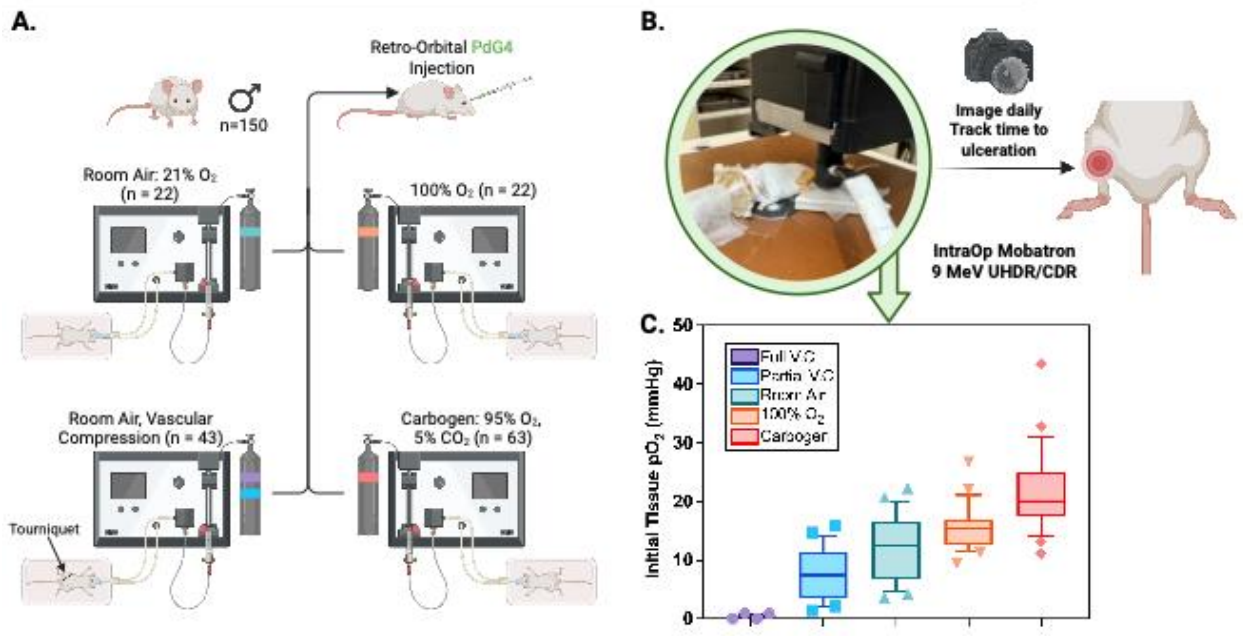

***Figure 1:*** *A) Study design, including population distribution, probe injection method, and oxygenation cohorts (room air, room air with vascular compression, 100% oxygen, and carbogen). Gas canisters are color-coded to match the legend and data in (C). B) Radiation delivery setup, showing mouse positioning photograph, and endpoint tracking. C) Box plots of initial tissue $pO_2$ at irradiation for each oxygen condition. Full vascular compression (V.C) with room air (purple), partial vascular compression with room air (blue), inhaled room air (green), inhaled 100% oxygen (orange), and inhaled carbogen (red).*

### *In Vivo* Oximetry Measurements

Real-time tissue oxygenation monitoring was accomplished using the phosphorescence-based oxygen probe Oxyphor PdG4 (Oxygen Enterprises, Philadelphia, PA). This dendritic probe operates via oxygen-dependent phosphorescence quenching and has undergone extensive validation for both *in vitro* and *in vivo* applications[30]. The Oxyphor PdG4 was administered through retro-orbital injection at 0.05 mL from 200 µM stock concentration, dissolved in phosphate buffer solution, approximately 60 minutes before radiation exposure to ensure adequate probe dispersion throughout the animal.

Oxygen measurements were captured using an OxyLED phosphorimeter system (Oxygen Enterprises, Philadelphia, PA). The system operated at a temporal resolution of 5 Hz (200 ms intervals), providing high-resolution oxygen dynamics during radiation delivery. To maintain measurement integrity while preventing radiation field interference, optical fibers were positioned at a 15 mm offset from the radiation field's center, oriented 45 degrees to the normal so as to allow adequate measuring of the irradiated tissue without impeding the electron beam, with the main instrumentation and data acquisition computer situated approximately 1 meter from the radiation source. Conversion of phosphorescence lifetime measurements to absolute $pO_2$ values was performed using pre-measured calibration constants for this batch of Oxyphor[30].

This measurement approach enabled quantification of radiation-induced oxygen dynamics in real time. Baseline tissue oxygenation was established by averaging pre-irradiation measurements per mouse, which were then subtracted from values obtained during and after radiation exposure[14]. The resulting differential oxygen measurements ($\Delta pO_2$) were normalized to radiation dose, yielding oxygen consumption g-values ($g_{O2}$), expressed as mmHg per unit dose delivered (mmHg/Gy).

**Statistical Analysis**

For the radiation-induced skin damage scoring, numerical values were evaluated daily with the standardized scoring system (Table 2) by trained observers. To address the issue of mice being removed from the study upon reaching endpoints, a last-observation-carried-forward (LOCF) approach was used, where the final observed skin score for each mouse was carried forward for all subsequent time points. This method preserved the impact of severe reactions in statistical analysis and prevented skewing of data as mice were removed from study. Using this dataset, a mixed-effects analysis was used to compare UHDR and CDR cohorts within each oxygen environment over the post-irradiation observation period. The tests included dose rate and time as fixed effects, with individual mice as random effects to account for correlation among repeated measures within each mouse. This approach appropriately handled the ordinal nature of the skin score data while accommodating the longitudinal study design. Separate tests were run for each oxygenation conditions to determine whether dose rate significantly influenced skin damage progression under specific oxygen environments. The null hypothesis tested was that dose rate did not affect the progression of radiation-induced skin damage under equivalent oxygenation conditions. Statistical significance was defined as p-value $\leq 0.05$.

To evaluate the difference in skin ulceration progression (score = 2 in Table 2), Kaplan-Meier plot analysis were used, displaying the proportion of mice that remained ulceration-free over the observation period. Separate curves were generated for UHDR and CDR treatment groups within each oxygenation condition. Statistical comparisons between dose rate groups were performed using Log-Rank (Mantel-Cox) tests, with p-value $\leq 0.05$ being considered statistically significant. This analysis allowed quantitative assessment of whether dose rate affects the timing and incidence of ulceration under controlled oxygen environments.

Comparison of oxygen consumption g-values and initial $pO_2$ values between mice that developed ulceration and those that remained ulceration-free was performed using Mann-Whitney U tests. The two experimental hypotheses ($H_1$) tested were that $pO_2$ and $g_{O2}$ are lower for UHDR-irradiated mice that did not develop ulcers through the duration of the study. Statistical significance was defined as p-value $\leq 0.05$.

## Results

### Skin $pO_2$ Observations in treatment groups

Tissue $pO_2$ values were varied through systematic induction of breathing gas or through clamping of blood flow, as illustrated in Figure 1. The induced ranges of skin $pO_2$, with mean and standard deviations illustrated in the graph in Figure 1C, were: (i) full vascular compression breathing room air, $pO_2 = 0.44 \pm 0.26$ mmHg, (ii) partial vascular compression breathing room air, $pO_2 = 7.4 \pm 4.3$ mmHg, (iii) freely breathing room air, $pO_2 = 11.9 \pm 5.6$ mmHg, (iv) freely breathing 100% oxygen, $pO_2 = 15.7 \pm 3.8$ mmHg, and (v) freely breathing carbogen, $pO_2 = 21.4 \pm 6.9$ mmHg. The total ranges were: full vascular compression 0-1.00 mmHg, partial vascular compression 1.4-16.0 mmHg, room air 3.6-22.3 mmHg, 100% oxygen 9.5-26.7 mmHg, and carbogen 11.1-43.4 mmHg.

These breathing and clamping groups were taken as treatment cohorts for the remaining skin damage response studies in order to systematically study the variation in skin damage.

**Skin Damage Outcome Difference**

Radiation-induced skin damage was monitored daily for 51 days post-irradiation, and mean skin scores over time for each cohort are shown in Figure 2 for each of the five oxygen conditions (panels A-E), with both CDR and UHDR groups on each of these. To analyze these data, a mixed-effects analysis with LOCF was used to reveal any significant differences between UHDR and CDR treatment groups under specific oxygenation conditions. Mice in the room air cohort (Figure 2C) exhibited significantly reduced skin damage following UHDR treatment compared to CDR (p-value = 0.001). A similar FLASH sparing effect was observed in the room air with partial vascular compression (Figure 2B) cohort (p-value = 0.005), and to a lesser extent in the 100% oxygen (Figure 2D) cohort (p-value = 0.03), where UHDR-treated mice had less severe reactions throughout the study.

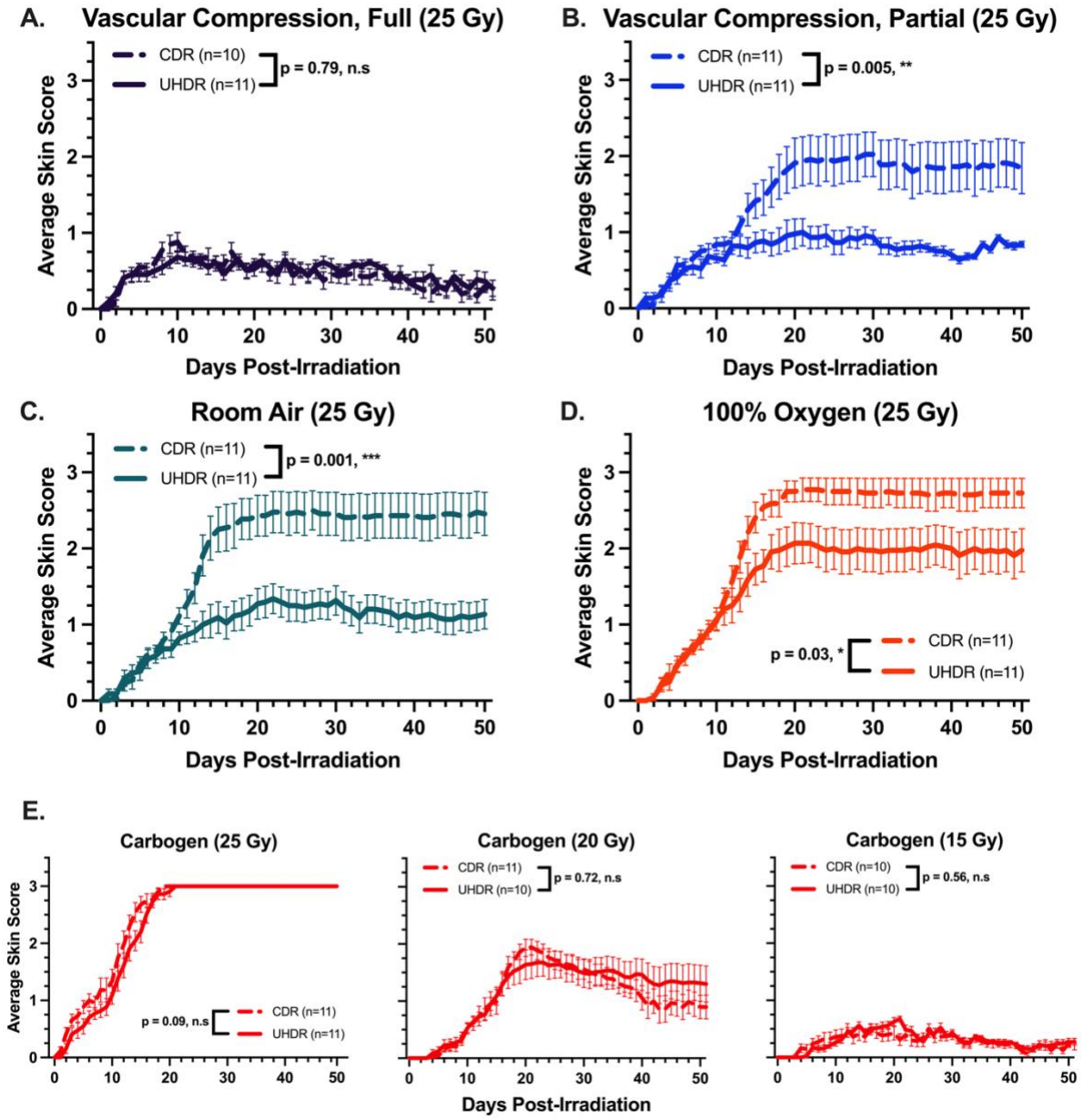


***Figure 2:*** *Average skin lesion progression daily for each oxygenation cohort. Solid lines represent UHDR data, and dashed lines represent CDR data. Error bars show standard error of the mean. Statistical significance of dose rate effect is indicated as ***(p-value ≤ 0.001), **(p-value ≤ 0.01), *(p-value ≤ 0.05), and n.s. (p-value > 0.05). A) Room air with complete vascular compression (purple, p-value = 0.79). B) Room air with partial vascular compression (blue, p-value = 0.005). C) Room air (green, p-value = 0.001). D) 100% oxygen (orange, p-value = 0.03). E) Carbogen inhalation for each of 25 (left), 20 (middle) and 15 (right) Gy dose cohorts (red, p-values = 0.09, 0.72, 0.56).*

In contrast, no significant differences were observed between UHDR and CDR groups in the carbogen breathing cohorts. Mice in the 25 Gy carbogen (p-value = 0.09) group developed comparable skin damage, though not a significant sparing effect, although the skin damage scores saturated potentially masking differences needed to determine if there was skin damage lessening or not. As a result, additional mouse cohorts were treated with carbogen but using dose levels of 20 and 15 Gy (Figure 2E, middle and right), to reduce the overall damage and yield lower skin damage scores. These did not show a significant FLASH skin sparing effect (p-values = 0.72 & 0.56) despite not reaching assay saturation.

**Skin ulceration endpoint**

The relationship between initial tissue oxygen tension ($pO_2$), oxygen consumption ($g_{O2}$), and ulceration is shown in Figure 3, with the raw data plotted in Figure 3 A, separating those animals that showed ulceration (red points) from those that did not (blue points). Among UHDR-irradiated mice, those that developed ulceration had significantly higher $g_{O2}$-values (0.17 ± 0.06 vs. 0.03 ± 0.07 mmHg/Gy, $p << 0.001$) and initial $pO_2$ levels (16.9 ± 5 vs. 3.8 ± 5.4 mmHg, p-value << 0.001) as compared to mice that remained skin ulcer-free, as shown in Figures 3 B and C. A linear regression fit of $g_{O2}$ and $pO_2$ yielded an $R^2$ value of 0.84.

Kaplan-Meier curves are shown in Figure 4, illustrating the differences in ulceration between UHDR and CDR groups across oxygenation conditions. In the normoxic (room air) cohort, UHDR reduced overall progression to ulceration compared to CDR (p-value = 0.01), with 8 out of 11 UHDR-treated mice remaining ulcer-free for the full study duration. In contrast, the median time to ulceration for CDR-treated mice was 15 days.

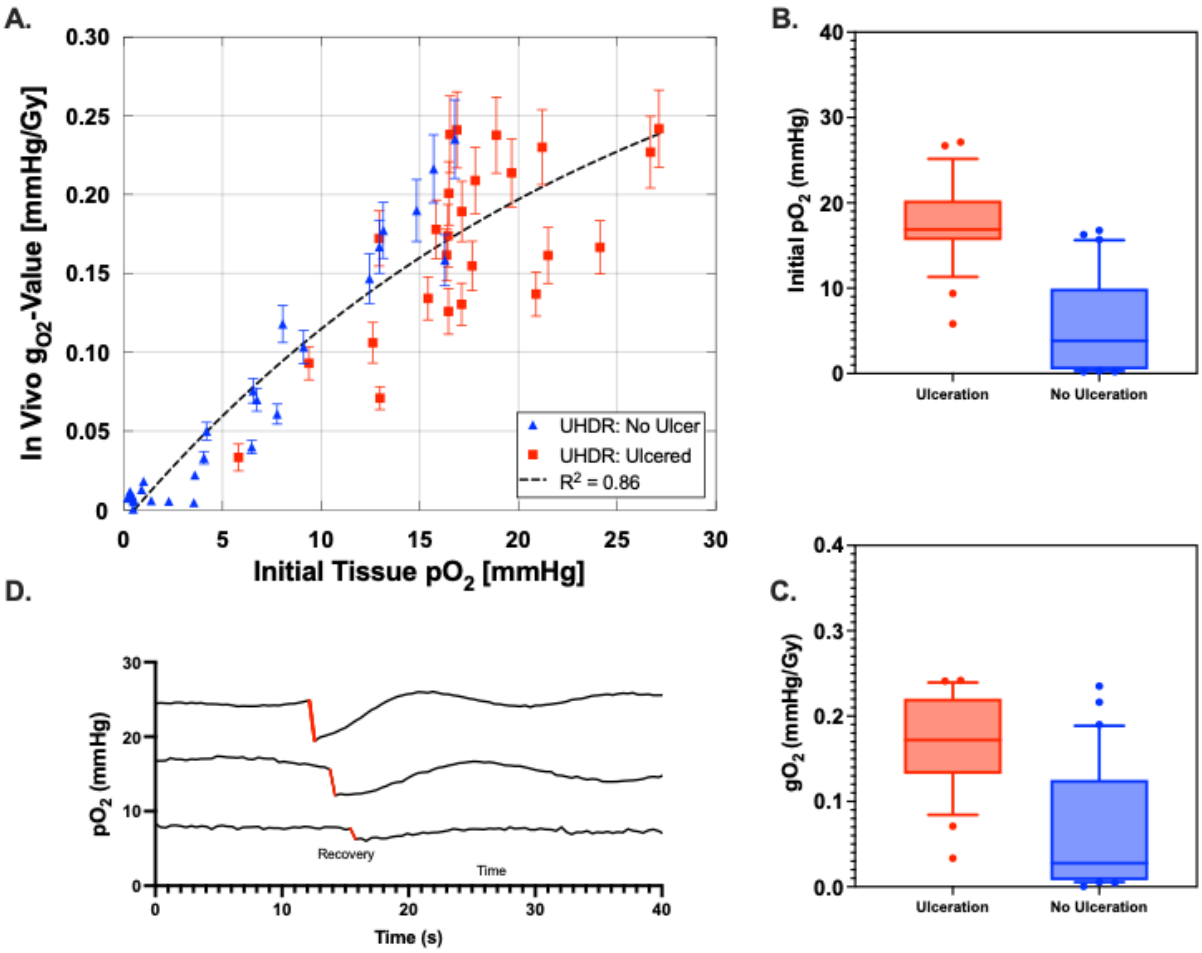


***Figure 3: A)*** *In vivo $g_{O2}$ measurements for UHDR-irradiated mice plotted against initial tissue $pO_2$. Each data point represents an individual mouse, with ulcered mice shown as red squares and non-ulcered mice as blue triangles. B) Box and whisker plots for initial $pO_2$ measurements between ulcerating (red) and non-ulcerating (blue) mice. C) Box and whisker plots for $gO_2$ measurements between ulcerating (red) and non-ulcerating (blue) mice. D) Sample recordings of murine skin $pO_2$ using the OxyLED system during UHDR irradiation, showing oxygen consumption (red) within the irradiated field.*

Despite a significant difference in skin scores (Figure 2B, p-value = 0.005), the partial vascular compression cohort did not show a statistically significant FLASH effect for ulceration-free progression (Figure 4B, p-value = 0.16). This likely reflects an overall reduction in radiation-

induced damage for CDR-treated mice in this cohort, leading to fewer ulceration events and in turn an underpowered log-rank test.

As opposed to the skin score analysis, no significant difference in ulceration-free progression was observed between UHDR and CDR groups in the 100% oxygen (Figure 4D, p-value = 0.15) cohort, and no difference was observed with the 25 Gy carbogen (Figure 4E, left, p-value = 0.46) cohort.

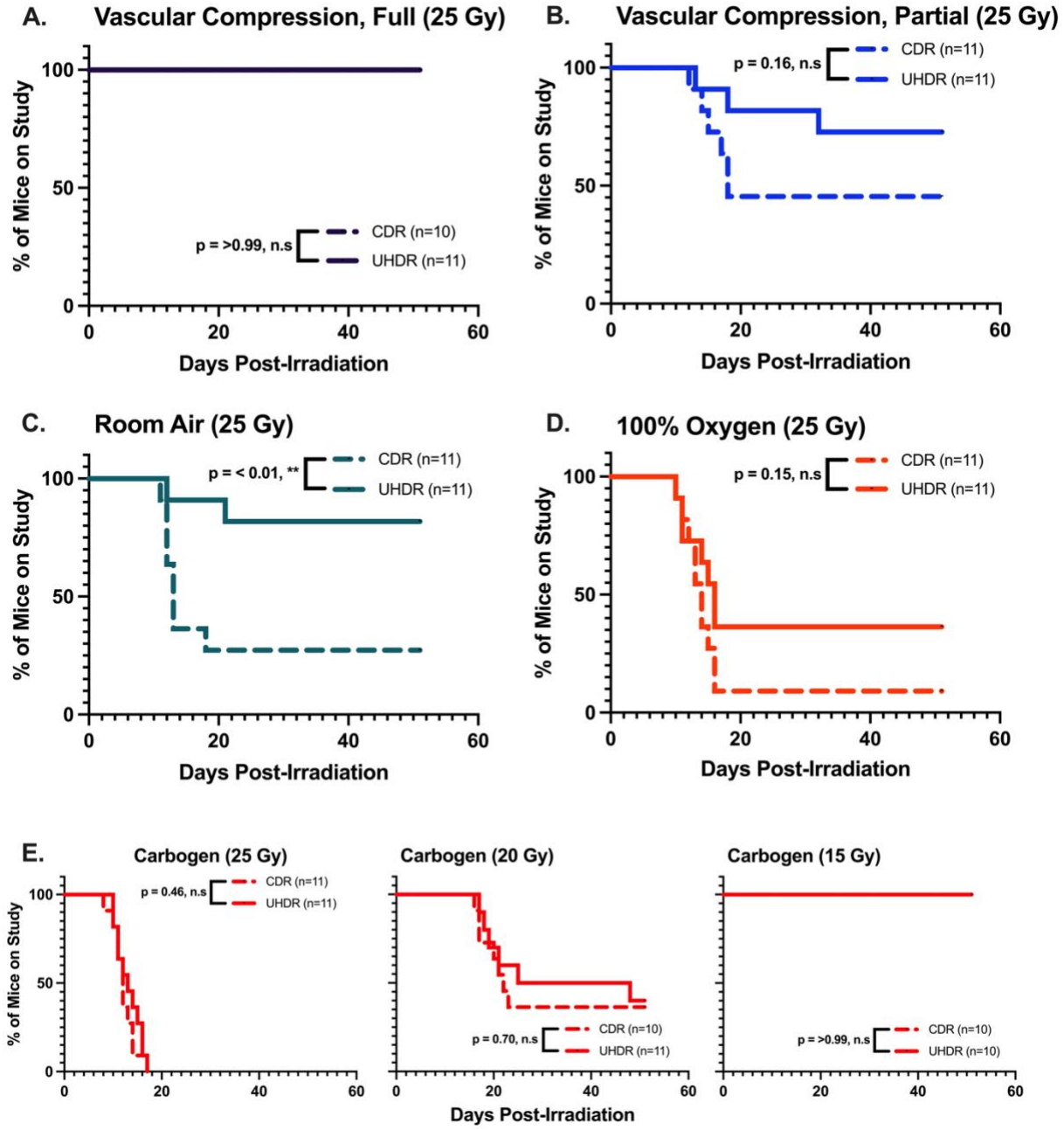


***Figure 4:*** *Kaplan-Meier curves for ulcer-free progression of mice in each oxygenation cohort. Solid lines represent UHDR data, and dashed lines represent CDR data. Statistical significance of dose rate effect is indicated as ** (p-value ≤ 0.01), * (p-value ≤ 0.05), and n.s. (p-value > 0.05). A) room air with full vascular compression (purple, p-value = >0.99). B) Room air with partial vascular compression (blue, p-value = 0.16). C) Room air (green, p-value*

*= < 0.01). D) 100% oxygen (orange, p-value = 0.15). E) Carbogen groups given 25 (left), 20 (middle) and 15 (right) Gy, respectively (red, p-value = 0.46, 0.70, >0.99).*

## Discussion

The FLASH effect of reducing damage with higher dose rate is now well established, but still the role of oxygen in terms of modulation and mechanism is unclear. In vitro data has been used to show that the FLASH damage reduction is present at mid-range oxygen values, disappearing at low oxygen and at high oxygen[31]. In vivo data has shown that UHDR radiation still effectively kills hypoxic tumors equivalent to CDR radiation[32], but it is not clear what happens when normal tissue is highly oxygenated or deoxygenated. Studies in rat brain showed the loss of the FLASH protective effect in the brain when supplemental oxygen was supplied to the anesthesia gas[33].

The present study was designed to test this phenomenon in a well-controlled in vivo model where oxygen could be systematically varied across the relevant range. The study results demonstrate that observable differences in damage score exist between CDR and UHDR in this *in vivo* skin model at the dose level evaluated, and we term this difference as FLASH skin sparing. The sparing is clearly dependent on initial tissue oxygen tension at the 25 Gy dose level investigated, being present in the ranges of oxygen found in the three conditions of room air, 100% oxygen and partial vascular compression ($pO_2 \approx$ 4-18 mmHg). However, when oxygen levels were modulated – either by complete anoxia (complete leg compression with ligation) inducing hypoxia ($pO_2 \approx 0$ mmHg) or hyperoxia, via carbogen inhalation (pO2 ≈ 15-28 mmHg), then the observation of FLASH tissue sparing was lost. Taken together, these finding suggest that baseline oxygenation level is likely a critical factor influencing not just the observation of the FLASH effect but also is likely at least part of the mechanism causing the FLASH effect.

Examining the results from the hyperoxia carbogen group, it was noted that the *in vivo* assay saturated at the highest damage level for 25 Gy, with all mice reaching the maximum skin score of 3.0 in both UHDR and CDR groups. The skin damage scoring scale has a maximum of 3.0, potentially masking differences between treatment groups under hyperoxic conditions, rendering the assay ineffective for these high damage levels. Thus, the lower dose groups of 20 and 15 Gy were included in the study under carbogen to determine if lower damage levels would allow observation of FLASH tissue sparing at hyperoxia $pO_2$ levels. However as seen in Figures 2 and 4, there was no significant FLASH sparing observable when these lower dose levels were used. It is possible that finer measures of damage reduction may observe some difference here, but the magnitude of sparing does not seem to match that seen under partial vascular compression, room air and 100% oxygen breathing conditions. Thus, these data are consistent with a hypothesis that excess oxygen negates the FLASH effect, suggesting that perhaps the FLASH effect itself is dependent upon some spatial or temporal limitation in oxygen at the irradiation site, consistent with previous studies[6,7,11,13,15,17,22,34,35]. It is still unresolved if the magnitude of radiolytic oxygen consumption is sufficient for full temporal depletion during the pulses, yet it seems most plausible that acute reduction in oxygen locally can alter the cascade of reactive oxygen species produced in each radiation pulse, thereby altering the nature of the radiation damage.

The link between oxygen tension and tissue damage is central to trying to understand the mechanisms of FLASH sparing, in terms of the underlying radiation chemistry. Under UHDR conditions, the relationship between $pO_2$ and tissue toxicity appears to be altered such that the expected severity of damage following 25 Gy is reduced for all but anoxic and hyperoxic tissues, showing the FLASH effect is present. In the UHDR setting, fixed levels of oxygen are consumed rapidly in formation of peroxyl radicals, thereby causing fixation of the radiation induced damage

in proteins and DNA. This occurs on a short time scale, where diffusion of oxygen back into the irradiated site occurs at a rate slower than consumption. It has been previously shown in vitro that UHDR irradiation reduces oxygen consumption as compared to CDR, thereby supporting the hypothesis that UHDR leads to less peroxyl damage to DNA. However, linking in vitro data and DNA data to in vivo observations of damage is inherently challenging and at best a correlative link can be established, rather than causative. Still the data here is consistent with the idea that oxygen is the mechanistic link leading to FLASH tissue sparing. Many studies have postulated and modeled transient oxygen depletion as a hypothesized mechanism for lower damage[36], although direct in vivo measurements of the level of consumption, here and in previous work[11,13,16], indicate that the level of oxygen consumed would not likely be sufficient for complete 'depletion' of local oxygen. Thus, there is not a clear explanation for this discrepancy and further data may be needed to determine if there are micro-localized depletions of oxygen or oxygen-related effects that are causative of reduced ROS damage in vivo.

Figure 2A, relative to the other panels, provides important insight into the role and potential mechanism of FLASH occurrence, based upon the loss of any sparing under anoxic conditions. In this completely hypoxic cohort, overall skin damage was reduced compared to room air, and there was no difference in damage between UHDR and CDR groups. This observation could suggest that whatever is causing the FLASH tissue sparing must rely upon oxygen being present in the tissue. Further work should evaluate whether the FLASH sparing effect persists at these anoxic oxygen levels, which may be relevant for future explorations into tumors and the FLASH normal tissue sparing effect.

The observations presented in Figure 3 provide a robust link between the radiation-induced oxygen consumption observed at UHDR and the biological FLASH effect. The relationship between radiation dose, dose rate, and tissue $pO_2$ suggests that the oxygen consumption g-value may serve as an important predictor for the onset of the FLASH effect. In fact, these findings build directly upon the concepts outlined in other publications in the broader FLASH field, which posit that oxygen chemistry under UHDR conditions is a determining factor for normal tissue sparing[12,14–17]. The data presented herein support the idea that proper initial oxygenation levels are required to harness the full benefits of FLASH radiotherapy.

Overall, these results suggest that there is a need to carefully control and report tissue oxygenation conditions when designing preclinical FLASH studies, and this can be as subtle as causing variations in tissue oxygen from breathing gas, animal temperature or mouse positioning (i.e. leg clamping). The evidence that FLASH-sparing is contingent on tissue oxygen tension not only provides insight into the chemical mechanisms of radiation damage but also has significant implications for the clinical translation of FLASH radiotherapy. By clearly establishing the necessary initial conditions for FLASH sparing, our work contributes to a more mechanistic understanding of how dose rate and oxygen interact to modulate tissue responses. Future studies might extend these observations to other tissue types and dose rate regimes, further exploring how oxygen consumption and radical chemistry at UHDR influence both acute and long-term normal tissue outcomes. Additionally, experiments should be conducted which investigate the role of modulating oxygen in the FLASH effect tumor response under UHDR, although current data suggests that hypoxic tumors are effectively treated as well with UHDR as they are with CDR[32]. It is not clear if this effect is purely due to oxygen radiation chemistry change, or a mixture of this with things like differences in endothelial cell damage, because of oxygen or simply blood flow.

Further it is possible that there could be hypoxia inducible factor signaling changes or immune cell treatment effects that alter the magnitude of FLASH sparing and oxygen variation is simply a biomarker of this. Understanding how UHDR effectively influences the OER across varied tissues will be critical for refining the mechanistic basis of the FLASH effect and for optimally translating these findings into clinical practice.

## Conclusion

This study demonstrated that in murine skin, tissue oxygen tensions achieved under room air anesthetic conditions are required for the most apparent observation of a skin FLASH sparing effect from UHDR versus CDR irradiation. Under partial vascular compression (hypoxic to mildly hypoxic), room air (mildly hypoxic) and 100% oxygen (normoxic to mildly hyperoxic) conditions, UHDR treatment resulted in a significant reduction in overall tissue damage compared to conventional dose rate exposures, and only the partial vascular compression condition achieved a significant reduction in overall ulceration. Hyperoxic skin with elevated local oxygen tension from carbogen breathing animals achieved maximal damage, and did not demonstrate any UHDR induced FLASH sparing, at any dose level studied. Anoxic skin from complete blood flow occlusion exhibited lower damage levels and no FLASH sparing effect. Measures of baseline $pO_2$ and radiolytic consumption, $g_{O2}$, during UHDR irradiation confirmed that they were related especially in non-ulcered skin at lower $pO_2$, and both were significantly related to the likelihood of skin ulceration.

These findings suggest that FLASH sparing which involves oxygen as a primary modulator, where the effect is only present for low levels of oxygen-mediated damage. Anoxic radiation damage

from non-ROS interactions appears to be unaltered by dose rate. The fact that FLASH sparing was also only present when there were lower levels of oxygen suggests that there is a modulating effect from oxygen that should be further investigated to see if it is a causal factor or a correlated biomarker.

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