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Scientific Article

Anesthetic Oxygen Use and Sex Are Critical Factors in the FLASH Sparing Effect

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Purpose: Ultra High Dose-Rate (UHDR) radiation has been reported to spare normal tissue, compared with Conventional Dose-Rate (CDR) radiation. However, important work remains to be done to improve the reproducibility of the FLASH effect. A better understanding of the biologic factors that modulate the FLASH effect may shed light on the mechanism of FLASH sparing. Here, we evaluated whether sex and/or the use of 100% oxygen as a carrier gas during irradiation contribute to the variability of the FLASH effect.

Methods and Materials: C57BL/6 mice (24 male, 24 female) were anesthetized using isoflurane mixed with either room air or 100% oxygen. Subsequently, the mice received 27 Gy of either 9 MeV electron UHDR or CDR to a 1.6 cm^2 diameter area of the right leg skin using the Mobetron linear accelerator. The primary postradiation endpoint was time to full thickness skin ulceration. In a separate cohort of mice (4 male, 4 female), skin oxygenation was measured using PdG4 Oxyphor under identical anesthesia conditions.

Results: Neither supplemental oxygen nor sex affected time to ulceration in CDR irradiated mice. In the UHDR group, skin damage occured earlier in male and female mice that received 100% oxygen compared room air and female mice ulcerated sooner than male mice. However, there was no significant difference in time to ulceration between male and female UHDR mice that received room air. Oxygen measurements showed that tissue oxygenation was significantly higher when using 100% oxygen as the anesthesia carrier gas than when using room air, and female mice showed higher levels of tissue oxygenation than male mice under 100% oxygen.

Conclusions: The skin FLASH sparing effect is significantly reduced when using oxygen during anesthesia rather than room air. FLASH sparing was also reduced in female mice compared to male mice. Both tissue oxygenation and sex are likely sources of variability in UHDR studies. These results suggest an oxygen-based mechanism for FLASH, as well as a key role for sex in the FLASH skin sparing effect.

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Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

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Introduction

Ultra High Dose-Rate (UHDR) radiation has been shown to spare normal tissue (FLASH effect) compared with Conventional Dose-Rate (CDR) radiation.[1-3] Still, important work remains to be done to optimize the magnitude and reproducibility of the FLASH effect.[4-7] Studies demonstrating a positive FLASH effect have used a wide variety of beam parameters, doses, animal models, and endpoints to optimize the effect.[8] For instance, using skin toxicity as the primary endpoint, Soto and colleagues showed FLASH sparing at 30-40 Gy but not at 10-20 Gy, whereas Duval and Aulwes et al demonstrated sparing at 25 Gy but not 30 Gy in the same animal model and under similar electron beam parameters.[9,10] Using a proton beam, Huang et al showed mouse strain-specific FLASH sparing at 27 Gy, but not at 15 Gy or 22 Gy.[11] On the other hand, studies that do not show FLASH sparing are sometimes considered to not have met the correct UHDR conditions.[12,13] This observation suggests that there are factors at play that modulate the FLASH effect that have not yet been identified. These variations, in part, stem from the lack of a solidified understanding of the factors underlying the FLASH mechanism and how it is modulated by different variables, such as the underlying biology of the model.

In CDR radiation therapy, it is widely believed that the presence of oxygen and generation of free radicals is necessary for optimal killing of cancer cells. This is supported by the observation that well-oxygenated tumors are 2 to 3 times more sensitive to radiation than hypoxic tumors.[14] This radio-sensitization effect caused by oxygen, the oxygen enhancement ratio (OER), is calculated as the ratio of the dose required to achieve the same biologic effect under hypoxic conditions to that under normoxic conditions. Notably, the OER varies by type of radiation therapy. For instance, the OER with proton therapy (OER, ~3), now believed to be similar for photon therapy, is about twice that of neutron or heavy-ion radiation therapy (OER, ~1.5).[15]

Despite the mechanism(s) of the FLASH sparing effect being an active topic of research, some of the most prominent hypotheses behind the mechanism of FLASH sparing continue to be oxygen based.[16] In fact, isolated studies have shown that hyperoxygenation and hypoxic conditions reduce or eliminate the FLASH effect.[17] The concept of OER is therefore paritculalry interesting in this context. If FLASH sparing is more sensitive to changes in tissue oxygen, then it would follow that different OERs apply to UHDR compared to CDR radiaiton. This would make tissue oxygenation an imporant source of variability in FLASH literature. Yet, most of the FLASH literature fails to report on or control for in vivo experimental variables that could meaningfully alter tissue oxygenation. These include type, concentration, and duration of anesthetic use; use of anesthetic oxygen; and physiological parameters, such as body temperature, respiratory rate, and sex.[18] This makes variations in tissue oxygen a likely and poorly controlled source of variability in UHDR studies. We therefore believe that further studies are needed that evaluate the FLASH sparing effect in dermal tissue under room air and 100% oxygen conditions, and between sexes, with direct tissue oxygen measurements.

In vivo measurements of tissue oxygenation are extremely challenging. While electrodes can provide precise readings, the measurement technique is damaging to the tissue, consequently not reporting the oxygenation of the normal healthy tissue and only providing a point sample measurement.[19] Other methods, like paramagnetic oxygen sensors (EPR oximetry), are invasive and require injection-site healing, and nuclear magnetic resonance relaxation methods can only report relative changes in averaged O_2 levels.[20] Therefore, optical methods that use a sensor (either a camera or fiber-optic fiber as the detector) to measure the quenching by oxygen of fluorescence probes have been used reliably to measure both the distribution and the average of the distribution, respectively, in vivo. The fluorescent probe PdG4 Oxyphor, specifically, has been used repeatedly to report extracellular oxygen levels in tissues, with the phosphorescence detector calibrated to extract absolute pO_2 readings from the lifetime-based quenching of PdG4.[21-23] Although injection of the fluorescent probe has the potential to disrupt tissue architecture, the small injection volume and high needle gauge are not likely to cause significant tissue damage.

Here, we hypothesized that anesthetic oxygen use and/or sex contribute to the variability of the FLASH effect and aimed to evaluate this hypothesis using mouse skin. We used subcutaneous injections of PdG4 Oxyphor to measure changes in tissue oxygenation under room air and 100% oxygen.

Methods and Materials

Skin irradiation

Animals

Forty-eight C57BL/6 mice (24 male, 24 female), ranging from 8 to 10 weeks of age, were acquired from Jackson Laboratories and allowed to acclimate for at least 3 days. Two days before the planned radiation delivery, the mice were tagged, and the right leg and leg area was shaved.

On the day of radiation delivery, the mice were anesthetized using isoflurane (induction: 3% isoflurane delivered at 500 mL/min for 3 min; maintenance: 1.5% isoflurane delivered at 100 mL/min) in either room air or 100% oxygen. Core body temperature was measured via a rectal probe and maintained at 37.5°. Mice were, on average, maintained on anesthesia for 10 min before irradiation.

Radiation delivery

A Mobetron intraoperative linear electron accelerator (IntraOp Inc, USA) was used to deliver a 27 Gy dose of 9 MeV CDR or UHDR radiation. A shielded collimator was used to collimate the radiation field to a 1.6 cm^2 diameter circular field. The right leg of prone mice was positioned on a 3D-printed holder, and a 1.6 cm^2 diameter area, centered at the midleg, was irradiated. For CDR delivery, the average dose rate was 0.17 Gy/s. For UHDR delivery, the average dose rate was 200 Gy/s, and the 27 Gy radiation was delivered in 2 pulses (2×3.16 μs at 120 Hz).

To calibrate dose delivery, the target CDR and UHDR dose was delivered to phantom (deceased) mice 24 h before the experiment, and dosimetry was verified by radiochromic film (EBT-XD) and thermolumiescent dosimeter. On the day of the experiment, quality assurance was conducted to ensure that both the UHDR and CDR beam output was within 5% of the expected value. The dose delivered to each animal was monitored by beam-current transformer measurements and verified by radiochromic film, calibrated from prior data.

Study endpoint

Mice were checked daily for skin lesions at the irradiation site by 2 independently trained investigators. On the day that a full thickness ulcer was noted, lesions were photographed. Discrepancies in the time to ulceration observed by the trained staff were resolved by a veterinary pathologist. Time to full thickness skin ulceration was used as the primary study endpoint for survival analysis. Mice that did not show lesions were monitored for 20 days postirradiation and censored at that time for survival analysis. All nonlesion mice were monitored for an additional 10 days beyond the study endpoint to ensure lack of lesion development. The study design is summarized in Fig. 1.

Measuring tissue oxygenation

Eight C57BL/6 mice (4 males, 4 females) were used over the course of 2 days; on the first day, the mice were anesthetized with 100% oxygen mixed with isoflurane, and on the second day, the mice were induced while

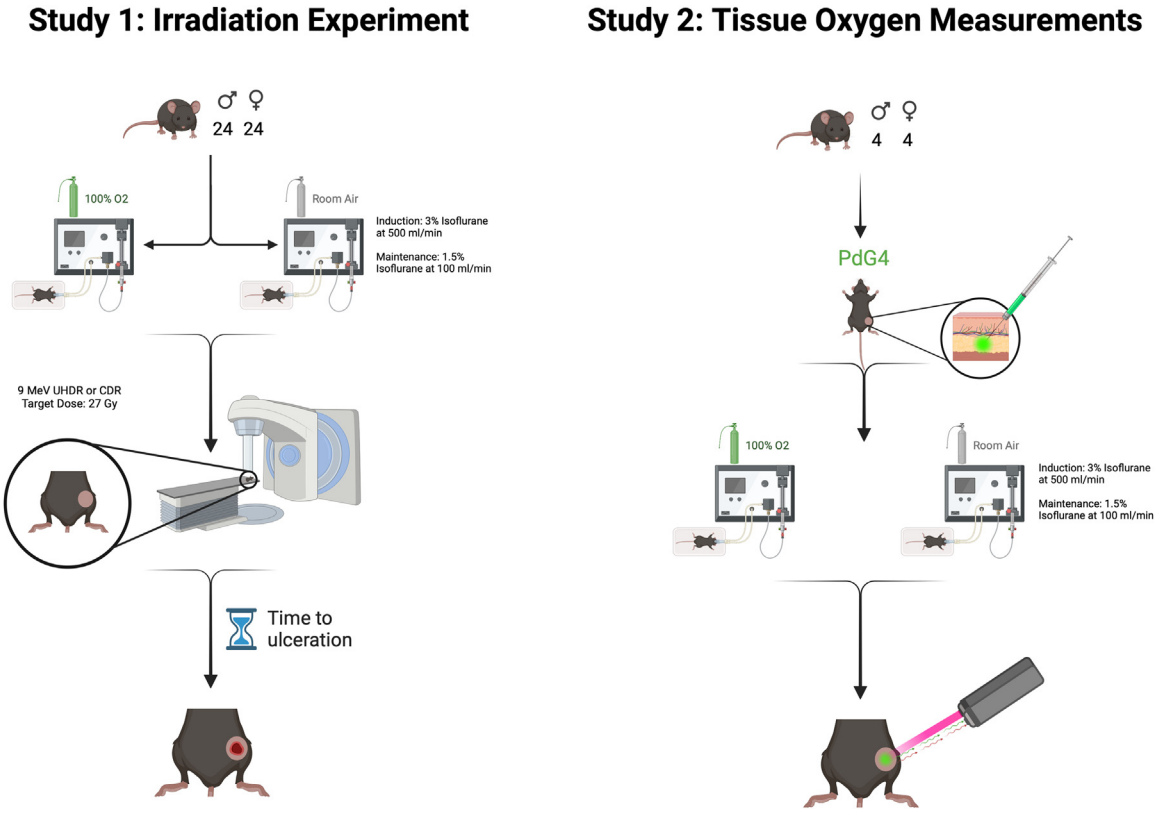

Figure 1 A visual demonstration of the study design. In study 1, 48 C57BL/6 mice (24 male, 24 female) were anesthetized using isoflurane delivered in 100% oxygen or room air and received 27 Gy of radiation to right leg skin using a Mobetron linear accelerator. Time to skin ulceration was measured as the primary endpoint. In study 2, 8 C57BL/6 mice (4 male, 4 male) received subcutaneous injections of PdG4 Oxyphor and were anesthetized similarly to study 1. An excitor-detector fiber-optic pair was used to read tissue oxygenation levels at the injection site. Figure created with BioRender.com.

breathing room air mixed with isoflurane. To simulate the breathing conditions of the mouse on the day of irradiation, each mouse was anesthetized as described in section 2.1. After a 3 min induction period, the left rear legs of the mice were shaved, and 0.05 mL of 100 μM PdG4 dissolved in phosphate-buffered saline was injected subcutaneously into the legs. The phosphorescence lifetime was read out by a commercial system (OxyLED, Oxygen Enterprises, Philadelphia, Pennsylvania) that was calibrated with the Stern-Volmer emission time constants for the PdG4 sample injected. From this system, the fiber pair contained a pulsed red (637 nm) excitation light and a collecting fiber, connected to an avalanche photodiode detector. This fiber was positioned approximately 5 mm from the skin surface, and oxygen pressure (mm Hg) was read out repeatedly for 10 min to sample the tissue pO_2 value. Core body temperature was maintained at 37.5° via external heating pad.

Statistical analyses

Using time to skin ulceration as the primary study endpoint, Kaplan-Meier survival curves were constructed for the irradiated mice. The log-rank test (Mantel-Cox) was used to compare survival data, and results were verified by Cox regression tests. The tissue oxygen measurement data were analyzed using independent sample *t* tests. All analyses were conducted using the SPSS Statistical Package (IBM Corp, USA). Graphics were constructed using GraphPad Prism (Prism Inc, USA) and BioRender software.

Results

Skin irradiation

The actual radiation dose delivered at the surface was 27 ± 1.5 Gy (5%). All mice were checked daily for skin lesion development at the irradiation site. As we have previously described, mice typically progress through dry and wet skin desquamation before full thickness ulceration/epidermolysis, though detection of wet and dry squamation can be variable. Therefore, time to full thickness skin ulceration was used at the primary endpoint.

Within the UHDR group, out of 12 mice breathing room air, 5 developed full thickness ulcerations. The remaining 7 mice did not develop lesions during the 20-day study period or 10-day follow-up period and were therefore censored from survival analyses at 20 days. Therefore, the median survival (time to skin ulceration) for room-air-breathing UHDR mice was greater than 20 days. On the other hand, out of the 12 mice breathing 100% oxygen, 11 developed ulcerations, and 1 did not, with a median time to skin ulceration of 12 days. This difference in time to skin ulceration was significantly different between the groups ($P < .05$).

Within the CDR group, out of 12 mice breathing room air, 11 developed ulcers, with a median time to ulceration of 9.5 days. Likewise, out of the 12 mice breathing 100% oxygen, 11 developed ulcers, but with a median time to ulceration of 15.5 days. This difference was not significantly significant.

Comparing the UHDR group to the CDR group, UHDR mice breathing 100% oxygen did not significantly differ from CDR mice breathing 100% oxygen in terms of the median time to skin ulceration (12 and 15.5 days, respectively). Conversely, UHDR mice breathing room air showed a significantly longer time to ulceration than CDR mice breathing room air, with a median time to skin ulceration of greater than 20 days and 9.5 days, respectively. These data are summarized in Fig. 2.

Separating the irradiation data by sex provided further insight into these differences. Within the UHDR group, female and male mice breathing room air did not differ significantly in terms of the time to skin ulceration, with a median time to ulceration of greater than 20 days compared with 18 days, respectively. In contrast, female mice breathing 100% oxygen developed skin ulcerations in a median of 11 days, which was significantly shorter than the median time to ulceration of male mice breathing 100% oxygen (15.5 days). This is summarized in Fig. 3A.

Looking at sex differences in the CDR group, female and male mice breathing either room air or 100% oxygen did not differ significantly in terms of the time to skin ulceration. These data are summarized in Fig. 3B.

Skin oxygenation measurements

The mean pO_2 of the leg in male mice breathing 100% oxygen (36 ± 7 mm Hg) was significantly higher than that in male mice breathing room air (21 ± 3 mm Hg). Similarly, the mean pO_2 of the leg in female mice breathing 100% oxygen (56 ± 11 mm Hg) was significantly higher than that in female mice breathing room air (26±4 mm Hg). Interestingly, the mean pO_2 for male mice breathing 100% oxygen was significantly different from that for female mice, as well; however, the difference in mice breathing room air was not significant. Results of tissue oxygenation measurements are summarized in Fig. 4.

Discussion

The literature on UHDR radiation and the FLASH effect shows wide variability in the presence and extent of normal tissue sparing, hinting that there may be unknown

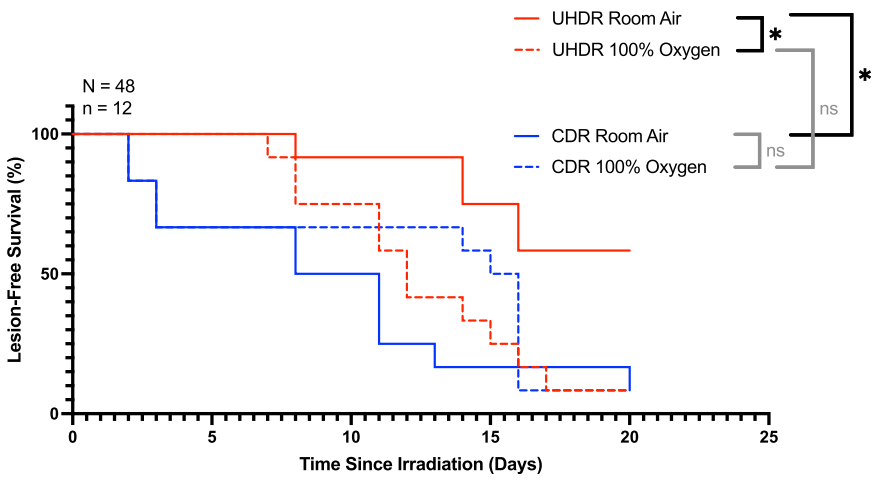

Figure 2 Kaplan-Meier curves demonstrating time to skin ulceration in leg skin of ultrahigh-dose-rate (UHDR) and conventional dose-rate (CDR) irradiated mice under room air and 100% oxygen conditions. Animals were removed from the study when a full thickness ulcer developed. Mice that did not show lesions after 20 days postirradiation were censored for survival analysis and monitored for an additional 10 days to ensure that no lesions developed. UHDR room air mice (median, ≥20 days) developed ulcers significantly later than both UHDR 100% oxygen mice (median, 12 days) and CDR room air mice (median, 15.5 days). UHDR 100% oxygen mice did not differ significantly from CDR 100% oxygen mice (median, 12 days). CDR room air mice and CDR 100% oxygen mice did not differ from each other in terms of the time to ulceration. *$P < .05$, ns = not statistically significant.

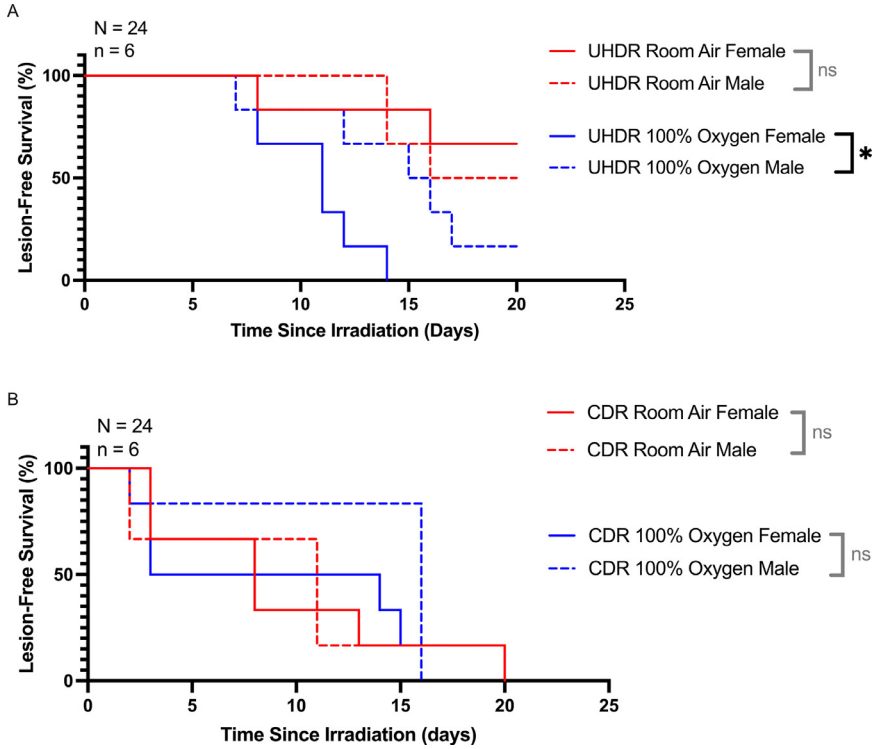

Figure 3 Kaplan-Meier curves demonstrating sex differences in time to skin ulceration in leg skin of ultrahigh-dose-rate (UHDR) and conventional dose-rate irradiated mice under room air and 100% oxygen conditions. (A) Male and female UHDR room air mice did not differ in terms of the time to skin ulceration. However, female mice in the UHDR 100% oxygen group developed ulcers earlier than male mice in the same group. (B) No significant differences were seen between male and female conventional dose-rate mice in either room air or 100% oxygen conditions. *$P < .05$, ns = not statistically significant.

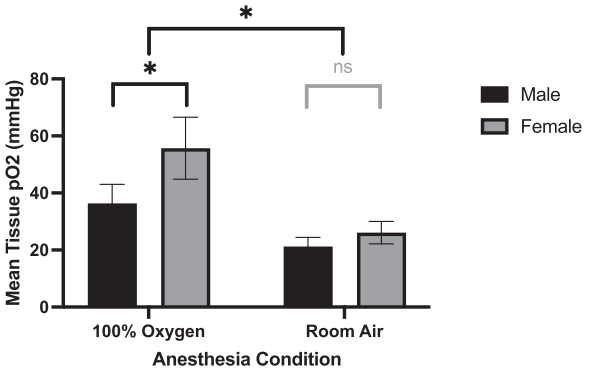

Figure 4 Direct tissue pO_2 measurements under room air and 100% oxygen conditions using PdG4 Oxyphor. Mice that received 100% oxygen had significantly higher tissue pO_2 levels than room air mice. The female mice that received 100% oxygen (mean, 56 mm Hg; SD, 11) had higher tissue pO_2 levels than the male mice that received 100% oxygen (mean, 36 mm Hg; SD, 7). $^*P < .05$, ns = not statistically significant.

sources of variability in in vivo studies. Here, we set out to determine the effect of oxygen use during anesthesia on FLASH sparing of normal skin tissue, supplemented by tissue oxygen measurements. We accomplished this by irradiating animals with CDR or UHDR radiation while delivering anesthesia using either 100% oxygen or room air as the carrier gas and measuring the time to skin ulceration.

Our results show that the time to ulceration in CDR radiation is not significantly affected by the oxygen concentration of the carrier gas. Conversely, UHDR-irradiated mice that received room air showed a significantly increased time to skin ulceration. Indeed, over half of the mice in the UHDR room air group did not show ulceration over the course of the study, compared with just 1 mouse in the UHDR 100% oxygen group. Comparing the UHDR- to CDR-irradiated mice, we observed no FLASH sparing effect in mice breathing 100% oxygen. On the other hand, significant sparing was seen between UHDR and CDR mice receiving room air as the carrier gas.

Breaking down our results by type of radiation and by sex, we showed a significantly reduced time to ulceration in female mice that received UHDR while breathing 100% oxygen compared with that in male mice. However, no sex difference was seen in UHDR mice that received room air or in CDR mice under either condition. This is an interesting finding, particularly considering the sex differences seen in our tissue oxygen measurements.

Using the PdG4 Oxyphor, we were able to repeatably measure oxygen levels in the mouse leg skin tissue. Although tissue oxygenation levels were similar between male and female mice under room air conditions, female mice breathing 100% oxygen showed significantly higher tissue pO_2 levels than male mice breathing 100% oxygen. This difference correlates with the difference seen in the time to ulceration between male and female mice in the UHDR 100% oxygen group, in which female mice (who showed higher tissue oxygen levels) ulcerated faster and were thus more radiosensitive. Though the mechanism behind the higher tissue oxygenation levels in female mice requires further investigation, we propose higher estrogen levels in female mice as a possible contributor. Estrogen is a potent angiogenesis factor, and it plays a critical role in both the maintenance and repair of dermal blood vessels.[24] If the dermis of a female mouse was more effectively vascularized, higher tissue oxygenation levels under anesthesia would be expected.

In summary, we made several important observations: First, the presence of 100% oxygen negated any FLASH sparing effect, primarily through its effect on UHDR-irradiated mice. Second, male and female mice are equally radiosensitive following CDR radiation under room air and 100% oxygen anesthesia conditions. Third, female mice are more radiosensitive than male mice when irradiated with UHDR radiaiton under 100% oxygen, but not under room air conditions. This However, once again, this difference was not seen in CDR-irradiated mice.

We suspect that other anesthetic conditions, such as depth of anesthesia (isoflurane/sevoflurane concentration, flow rate, and length of induction and maintenance) and physiological parameters like respiratory rate and body temperature, are likely to have a pronounced effect on tissue oxygen levels, and consequently, the extent of sparing in UHDR radiation. For instance, in a current, separate study, we have found that maintaining animals at 3% isoflurane results in significantly lower tissue oxygenation levels than those observed at the 1.5% used in this study.

Further investigation into the source of the sex differences in FLASH sparing and tissue oxygenation is also needed. Skin as a model for FLASH effects may also be problematic because of the variability in control over skin oxygenation between animals, sexes, and physiological conditions, although at the same time, it presents as an interesting model in which factors affecting the mechanism of FLASH may be teased out. This work makes clear the need for careful control and reporting of these variables in future UHDR in vivo studies.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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