Modeling the impact of tissue oxygen profiles and oxygen depletion parameter uncertainties on biological response and therapeutic benefit of FLASH

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Abstract

Ultra-high dose rate (FLASH) radiation has been reported to efficiently suppress tumor growth while sparing normal tissue, however, the mechanism of the differential tissue sparing effect is still not known. Oxygen has long been known to profoundly impact radiobiological responses, and radiolytic oxygen depletion has been considered to be a possible cause or contributor to the FLASH phenomenon. This work investigates the impact of tissue pO₂ profiles, oxygen depletion per unit dose (g), and the oxygen concentration yielding half-maximal radiosensitization (k) in tumor and normal tissue. We developed a model that considers the dependent relationship between oxygen depletion and change of radiosensitivity during FLASH irradiation. Cell survival was calculated based on the LQ-L model and the radiosensitivity related parameters were adjusted while delivering fractional doses of FLASH irradiation. The model reproduced published experimental data that were obtained with different cell lines and oxygen concentrations, and was used to analyze the impact of parameter uncertainties on the radiobiological responses. This study expands the oxygen depletion analysis of FLASH to normal human tissue and tumor based on clinically determined aggregate and individual patient pO₂ profiles. The results show that the pO₂ profile is the most essential factor that affects biological response and analyses based on the median pO₂ rather than the full pO₂ profile can be unreliable and misleading. Additionally, the presence of a small fraction of cells on the threshold of radiobiologic hypoxia substantially alters biological
response to FLASH irradiation. We found that an increment in the $k$ value is generally more protective of tumor than normal tissue due to a higher frequency of lower pO$_2$ values in tumors. Variation in the $g$ value affects the dose at which oxygen depletion impacts response, but does not alter the dose dependent response trends, if the $g$ value is identical in both tumor and normal tissue. The therapeutic efficacy of FLASH oxygen depletion is likely patient dependent and beneficial in a minority of breast cancer cases, however, in a subset of well oxygenated tumors, a therapeutic gain may be realized due to induced normal tissue hypoxia.

1 Introduction

In 2014, Favaudon et al. reported that a single dose of 20 Gy radiation with electrons administered to the thorax of rats at a mean dose rate $\geq$ 40 Gy/s (FLASH irradiation) resulted in “no lung complications”, whereas 15 Gy administered at a conventional (CONV) dose rate ($\leq$ 0.03 Gy/s) lead to significant lung fibrosis (Favaudon et al., 2014). Additionally, FLASH and CONV irradiation equally suppressed tumor growth (Favaudon et al., 2014; Diffenderfer et al., 2020; Levy et al., 2020). The sparing of normal rather than tumor tissue has given rise to significant clinical interest in extremely high dose-rate radiation for the treatment of cancer as well as investigations into the potential mechanism of the FLASH effect. Although several hypotheses have been advanced to explain the normal tissue sparing effect of FLASH irradiation, including rapid radiolytic oxygen depletion, changes in the production and processing of reactive oxygen species, immune response, microenvironmental factors, and others, the mechanism(s) of tissue sparing have not yet been resolved (Adrian et al., 2020; Wardman, 2020; Pratx and Kapp, 2019b; Spitz et al., 2019; Wilson et al., 2020; Jin et al., 2020; Petersson et al., 2020; Labarbe et al., 2020; Zhou, 2020). Additionally, most but not all studies have reported normal tissue sparing at FLASH dose rates (Venkatesulu et al., 2019; Smyth et al., 2018), and Adrian et al reported a FLASH effect in several tumor cell lines in vitro (Adrian et al., 2021).

It has long been known that both CONV and FLASH irradiation deplete dissolved oxygen in aqueous solutions. The principal mechanism of oxygen depletion, i.e., the binding of oxygen with primary or secondary radical products of water radiolysis suggests that radiation induced hypoxia is likely to occur in cells and tissues if the rate of intracellular oxygen depletion exceeds the rate of oxygen resupply from the extracellular environment, regardless of whether the cells and tissues
are normal or malignant. Given the pronounced radiosensitizing effect of oxygen, the therapeutic efficacy of FLASH irradiation will thus likely be impacted by radiation induced hypoxia in tumor and normal tissue. It is of note that the earliest and perhaps most frequent validation of FLASH tissue sparing has been reported for skin, which is known to be on the threshold of radiobiologic hypoxia in its normal state (5-10 mmHg oxygen) (Vozenin et al., 2019; Wilson et al., 2020; Field and Bewley, 1974; Soto et al., 2020; Bourhis et al., 2019; Bedogni and Powell, 2006).

Biologic effects of radiation principally arise from damage to DNA. This damage may result from direct interactions of ionizing radiation with DNA, or indirectly from the interaction between chemical products generated by the radiolysis of water and DNA. Most indirect lethal damage is caused by the hydroxyl radical \( \cdot \mathrm{OH} \). The resulting DNA\(^{-} \) radical may either be restored to its undamaged state by hydrogen donation, primarily by amino thiols such as glutathione, cysteine, and cysteamine, or oxidized by oxygen, leading to the formation of peroxides which “fix” the DNA damage, i.e., make the damage permanent (Alexander, 1962; Koch, 1988; Bump et al., 1992). The fate of the DNA\(^{-} \) radical is thus dependent on competition between oxygen for damage fixation, and thiols for damage repair. The oxygen-thiol competition model for fixation or restoration of the DNA radical as well as competing radiochemical processes in mammalian cells has been validated and summarized by Koch (Koch, 1988). In both bacteria and mammalian cells, the oxygen concentration needed to achieve half-maximum sensitization, which is usually denoted as \( k \), is increased in the presence of added thiols and decreased upon thiol depletion (Koch, 1988; Dewey, 1963).

At sufficiently high doses and dose-rates, when the rate of cellular oxygen depletion exceeds the rate of oxygen diffusion into cells, both bacteria and mammalian cells exhibit a pronounced decrease in sensitivity to radiation, and the dose at which the sensitivity to radiation decreases is directly dependent on the initial oxygen concentration (Dewey and Boag, 1959; Weiss et al., 1974; Nias et al., 1969; Epp et al., 1972). While these quantitative studies and results have largely been pioneered and demonstrated in bacteria and mammalian cells in vitro, the impact of radiobiologic hypoxia on the response of tumors, normal tissues and spheroids also yield oxygen enhancement ratios (OER) of approximately 2.5-3.0 (Suit and Maeda, 1967; Khan et al., 2021; Wright and Bewley, 1960; Wright and Batchelor, 1959). In short, small naturally occurring or induced changes in oxygen status may significantly impact cell and tissue response. This effect becomes especially
significant in the context of stereotactic body and FLASH irradiation, which utilize doses in the range of 10-20 Gy per fraction.

The extent to which FLASH oxygen depletion impacts tissue response will depend on pretreatment tissue pO2, oxygen depletion per unit dose, total dose and the oxygen concentration at which half-maximal sensitization occurs. In this study, we examined two descriptors of tissue oxygenation, i.e., median tissue pO2 and complete pO2 tissue profiles, and the impact of the reported ranges and uncertainties in the aforementioned parameters on FLASH oxygen depletion and the resultant change in cell response. The results show that the therapeutic efficacy of FLASH is likely patient dependent. We identify circumstances under which FLASH oxygen depletion could be of therapeutic benefit or deficit.

2 Methods

2.1 Modeling the impact of FLASH oxygen depletion on cellular response

Based on decades of evidence, this study assumes that the oxygen concentration of tumor and normal tissue is a determinant of response to radiation. To evaluate the potential impact of oxygen depletion, including uncertainties in the oxygen depletion (g) per unit dose and the oxygen concentration at which the OER reaches half-maximum (k), cell surviving fractions (SF) were calculated based on the linear quadratic-linear (LQ-L) model (Astrahan, 2008):

\[
\ln(SF) = -(\alpha D + \beta D^2), \quad \text{for } D \leq D_T
\]  
\[
\ln(SF) = -(\alpha D_T + \beta D_T^2) - \gamma (D - D_T), \quad \text{for } D > D_T
\]

\(\alpha\) and \(\beta\) are inactivation parameters which characterize cell and tissue response to radiation, \(D_T\) is the transition point at which the SF curve becomes linear, \(\gamma\) is the log cell kill per Gy in the linear portion of the survival curve as determined by the slope of the line tangent to the LQ curve at dose \(D_T\).

To estimate the impact of oxygen on cell response, the method proposed by Carlson et al. was used to modify the parameters of \(\alpha\) and \(\beta\) (Carlson et al., 2006). i.e.:
\[ \alpha_{\text{aerobic}} = \alpha_{\text{anoxic}} \times \text{OER} \]  \hfill (2)

\[ \left(\alpha/\beta\right)_{\text{aerobic}} = \left(\alpha/\beta\right)_{\text{anoxic}} / \text{OER} \]  \hfill (3)

\[ \text{OER} = \frac{k + m \times [O_2]}{k + [O_2]} \]  \hfill (4)

OER was calculated with the empirical function proposed by Alper and Howard (Alper and Howard-Flanders, 1956). \( m \) is the maximum OER and \( k \) is the oxygen concentration (mmHg) at which the OER is equal to half of its maximum value. \([O_2]\) is the oxygen concentration (mmHg). In this study, \( m \) was assumed to be 3. The transition point \( D_T \) for cells with different oxygen concentration in the LQ-L model was calculated with:

\[ D_T|_{\text{aerobic}} = D_T|_{\text{anoxic}} / \text{OER} \]  \hfill (5)

### 2.2 Model evaluation and processing of tissue pO2 profiles

To evaluate the validity of the model we determined whether it predicted the experimental results reported by Ling et al. (Ling et al., 1978) and Michaels et al. (Michaels et al., 1978), Figure 1. These investigators placed attached CHO cells coated with a thin film of medium into a humidified 100% N\(_2\) environment or an environment containing 0%, 0.21% and 0.44% oxygen in N\(_2\). The cells were then exposed to single dose irradiation of 3 ns duration. During the 3 ns dose, cells were killed and oxygen was depleted. At a dose which deleted all oxygen, the cell sensitivity was identical to the sensitivity of cells irradiated under 100% N\(_2\) conditions. To quantify the impact of oxygen depletion during the 3 ns pulse on the radiation sensitivity parameters \( \alpha \), \( \beta \) and \( \gamma \), we utilized the Alper and Howard-Flanders competition model (Alper and Howard-Flanders, 1956) and updated the cell surviving fraction after every fractional dose of 1 Gy with 0 time elapsing between each fractional dose. Practically, this was implemented via the following steps:

1. Deliver the \( n^{th} \) fractional dose and decrease the intracellular oxygen concentration by the value of \( g \);
2. Calculate the OER\(_{n}\), and the OER\(_{n}\) adjusted \(\alpha_n, \beta_n, \gamma_n\), and \(D_{Tn}\) according to Eq.1-5, and calculate \(SF_n(n - 1 \text{ Gy})\) and \(SF_n(n \text{ Gy})\) according to Eq.1 with the updated parameters for this n-th pulse.

3. Calculate the fractional decrease in the surviving fraction \(d_{SF|n} = 1 - \frac{SF_n(n \text{ Gy})}{SF_n(n - 1 \text{ Gy})}\). The SF after the \(n\)th fractional dose is then calculated in a recursive manner for each fractional dose: \(SF(n \text{ Gy}) = SF(n - 1 \text{ Gy}) \times (1 - d_{SF|n})\)

The same oxygen depletion and radiation survival model (LQ-L) was applied to more complex tissue pO\(_2\) profiles, i.e., containing well oxygenated foci as well as low pO\(_2\) foci in the same tissue. Briefly, the percent cells or foci within a pO\(_2\) range such as 0-2.5, 2.5-5, 5-7.5 mmHg up to the highest recorded pO\(_2\) value is processed in the same way as described for cells. All cells in each bin are assumed to be at the same pO\(_2\) i.e., the mid pO\(_2\) value of each bin e.g., 1.25 mmHg in the 0-2.5 mmHg bin. The sum of the surviving fractions in each 2.5 mmHg bin was then calculated. As each 1 Gy fractional dose depletes oxygen, alpha and beta values are accordingly recalculated for each additional 1 Gy fractional dose.

2.3 Application of the model to human tissue; methodology and assumptions

A treatment site (breast) for which substantial pO\(_2\) data is available for both tumor and normal tissue (Vaupel et al., 1991) was selected to evaluate the effects of FLASH vs. CONV irradiation in human tissue with a heterogeneous pO\(_2\) distribution. Vaupel et al. (Vaupel et al., 1991) obtained aggregate normal breast pO\(_2\) profiles of \(N=16\) patients, \(n=1009\) evaluated foci along with breast tumor pO\(_2\) profiles in 15 of the same \(N=16\) patients, \(n=1068\) foci, and the pO\(_2\) profile of two individual patient’s breast tumor assessed by the Eppendorf polarographic system. The pO\(_2\) profiles of normal human brain and subcutis were also extracted from Vaupel et al. for analysis (Vaupel et al., 1989). The pO\(_2\) profiles were extracted using the GetData graph digitizer (http://getdata-graph-digitizer.com/), and presented as the relative frequency of tissue in each 2.5 mmHg pO\(_2\) bin (Figure 2).

The SF responses of normal breast and breast tumor were calculated by the following method:

1. For CONV irradiation, it was assumed that the oxygen supply exceeded the rate of oxygen depletion and the tissue pO2 profile was unchanged.
2. For FLASH irradiation, the pO$_2$ profile was shifted by $g \times 1$ Gy after each 1 Gy fractional dose; the parameters OER, and OER adjusted $\alpha, \beta, \gamma, D_T$ values were updated in each bin to calculate the SF using the fractional dose method described above.

3. Radiolytic oxygen depletion is regional and equally applies to both the cellular and extracellular compartments. It is assumed that intracellular and extracellular oxygen depletion exceeds the rate of oxygen resupply from the nearest oxygen rich precapillary arterioles and capillaries.

2.4 Parameter values in the model

To reproduce the experimental data reported by Ling et al. (Ling et al., 1978) and Michaels et al. (Michaels et al., 1978) the parameter values $\alpha_{\text{anoxic}} = 0.0156$ and $\beta_{\text{anoxic}} = 0.0071$ were determined by fitting the SF data measured under anoxic condition from the experimental N$_2$ SF curve with the LQ-L model. $g = 0.275$ mmHg/Gy was adopted as their data indicate 12 Gy depletes 0.44% oxygen (3.3 mmHg), similar to the average $g$ value reported for mammalian cells, (Supplemental material section 1). The estimated 12 Gy transition dose $D_T$ was determined based on the experimental SF curve shape, i.e., the point at which the dose response curve became linear as reported by Michaels et al. (Michaels et al., 1978).

For the analysis of normal human breast and breast tumor tissues, the $\alpha$ and $\beta$ values for breast tumor were adopted from (Gould and Howard, 1989) with $\alpha = 0.374, \beta = 0.0251$. Late skin response ($\alpha = 0.0432, \beta = 0.0227$) was considered as a surrogate for normal breast (Kehwar, 2005). $D_T|_{\text{aerobic}}$ was set as 10 Gy for both normal breast and breast tumor.

Differences in the intracellular concentration of aminothiols between tissue types as well as in vitro and in vivo, have been reported and shown to impact the value of $k$. $k = 3.8$ mmHg was used for the calculation of cell SF, while the range of 3.8-15 mmHg was considered to evaluate the impact of the uncertainty of $k$ on cell survival (Bump et al., 1992; Koch, 1988; Bergsten et al., 1990; Dewey, 1963).

The parameter $g$, that is oxygen depletion per unit dose, is one of the most impactful factors in FLASH oxygen depletion. In this work we evaluated the minimum and maximum reported values of $g$, i.e., $g = 0.19$ and $0.71$ μM/Gy (0.15 and 0.56 mmHg/Gy) to investigate the impact of the $g$ value uncertainty on cell survival. A detailed summary of previously reported value of $g$ can be
found in the supplementary material section 1. We further used the mean reported \( g \) (0.45 \( \mu \)M/Gy, i.e., 0.36 mmHg/Gy) as the FLASH oxygen depletion rate for other calculations.

For determination of the 95% confidence level of the number of recorded pO\(_2\) values in each 2.5 mmHg bin, the percent observations in each bin were multiplied by the total number of observations for all bins. The values shown are binomial exact estimates.

3 Results

3.1 Evaluation and validation of the oxygen depletion and LQ-L models

Figure 1, panels a and b show the results of in vitro studies of Ling et al, 1978 and Michaels et al 1978. Cells were equilibrated with a gas phase environment of 100% N\(_2\), 0.21% and 0.44% O\(_2\). The cells were exposed to doses of radiation as previously described. The surviving fractions of cells irradiated under N\(_2\), 0.21% and 0.44% O\(_2\) conditions are indicated by the symbols. The oxygen depletion and LQ-L predicted surviving results are indicated by the dashed curves. Figure 1a also shows that a factional dose of 1 Gy or 0.1 Gy yields similar results, indicating that the 1 Gy factional dose is sufficient to describe the oxygen depletion process and cell kill which occur over the same time scale. Essentially identical results were obtained in an examination of the predicted vs. observed results in HeLa cells (supplementary Figure S1).

![Graph showing survival fraction vs. dose for different oxygen conditions and fractional doses.](image)
Figure 1. The surviving fraction of Chinese hamster ovary (CHO) cells after 3 ns electron FLASH irradiation under different initial oxygen concentrations. The data of cells equilibrated with nitrogen is indicated by black dots and circles; 0.21% and 0.44% oxygen by blue and red triangles. Experimental data are extracted from Ling et al. (Ling et al., 1978) and Michaels et al. (Michaels et al., 1978). The dashed lines represent the calculated results obtained with the fractional dose method for $\alpha_{anoxic} = 0.021$, $\beta_{anoxic} = 0.0071$ and $D_T = 12$ Gy.

3.2 Quantifying tissue oxygenation: median pO2 values are not appropriate for predicting the impact of FLASH irradiation.

Tissue oxygenation is frequently characterized by a single parameter such as median pO2. However, modulation of radiation sensitivity occurs over a narrow pO2 range, i.e., < 15 mmHg, and most prominently over the 1 to 7 mmHg range. Median or mean pO2 does not reflect the percent of tissue in the 0-15 mmHg range, and more specifically, the fraction of cells in the 0-2.5 mmHg range, 2.5-5, 5-7.5 mmHg range, etc. Reported FLASH oxygen depletion values (0.19 - 0.71 μM/Gy) suggest that FLASH irradiation may reduce the pO2 of tissue on the threshold of radiobiologic hypoxia, into a substantially radiation protected pO2 environment. Based on median pO2 values, this induced hypoxic radioprotection would generally be thought to be highly unlikely.

Figure 2 compares the response of normal human brain, subcutis (Vaupel et al., 1989) and breast cancer (Vaupel et al., 1991) to CONV vs. FLASH dose rate irradiation for median pO2 values (red lines) and full pO2 profiles (black lines). The most prominent features are that median tissue pO2 value does not reflect the presence and frequency of low pO2 values (e.g., compare the fraction of low pO2 values in breast cancer, median pO2 = 30 mmHg, with the low pO2 fraction in normal brain median pO2 = 24 mmHg). Similarly, median pO2 values do not reveal the large response difference of tissue which contain a large fraction of hypoxic cells (breast cancer) when exposed to CONV vs. FLASH irradiation (panel c). Neither do median pO2 values suggest that tissue such as normal brain may benefit from FLASH vs. CONV irradiation, or that the response of breast tumor which has a relatively high median pO2 and large percentage of cells at very low pO2, exhibits a differential response when exposed to FLASH vs. CONV dose rate irradiation. To
summarize, median or average tissue pO2 is an unreliable and likely misleading parameter of tissue response to FLASH irradiation.

Figure 2. The pO2 profile of (a) brain, (b) subcutis, and (c) breast cancer, data adopted from references (Vaupel et al., 1991; Vaupel et al., 1989)] and the (d-e) SF curves calculated with the full pO2 profile or median pO2. \( \alpha_{\text{Brain}} = 0.0499, \beta_{\text{Brain}} = 0.0238 \) (Kehwar, 2005); \( \alpha_{\text{Subcutis}} = 1.13, \beta_{\text{Subcutis}} = 0.0 \) (Dahlberg et al., 1993) ; \( \alpha_{\text{Breast Cancer}} = 0.374, \beta_{\text{Breast Cancer}} = 0.0251 \) (Gould and Howard, 1989)

3.3 Impact of the pO2 profile uncertainty

Figure 3 shows the pO2 profile of aggregate normal breast, aggregate breast tumor and patient specific breast tumors before and after 20 Gy FLASH irradiation, along with the 95% confidence intervals of the percent of cells in each bin. The final frequency of tissue in each 2.5 mmHg bin was obtained by following 3 steps after each 1 Gy fractional dose: ① shift the pO2 profile by \( g \times \) fractional dose; ② calculate the surviving fraction in each bin using the adjusted \( \alpha, \beta, \gamma, D_T \) values; ③ repeat the process until a preset total dose is delivered; ④ normalize the pO2 profile so that the
sum of frequencies in all bins equals 100% (A graphic description is provided in supplementary Figure S2).

Aggregate breast tumor, panel b, blue line, is substantially more hypoxic than normal breast prior to irradiation, panel a blue line, with approximately 15% of pO2 values being < 5 mmHg, and 30% < 10 mmHg. Twenty Gy FLASH irradiation further reduces the pO2 by approximately 7.2 mmHg and 99.36 % of cells surviving 20 Gy reside in the 0-2.5 mmHg bin as indicated by the orange curve. However, substantial intertumoral pO2 heterogeneity is also suggested by the approximately 50% of pO2 values greater than 20 mmHg. This is seen in the breast cancer pO2 profiles of patients A and B, Figure 3c and 3d. None of the tumor cells of patient A exhibits radiobiologic hypoxia after 20 Gy FLASH irradiation. In contrast, in patient B, approximately 16% of all cells are between 0 and 2.5 mmHg, and approximately 50% of cells’ pO2 values are < 5 mmHg prior to irradiation. Following 20 Gy irradiation, 99.85% of all surviving cells reside in the 0-2.5 mmHg pO2 bin category. The very significant increase in the fraction of surviving cells in the 0-2.5 mmHg bin following 20 Gy FLASH irradiation, is due their greater radiation resistance and to the shift of cells at higher pO2 to the lower pO2 bin due to oxygen depletion. In aggregate normal breast and breast cancer of patient A, 20 Gy FLASH oxygen depletion is insufficient to reduce the pO2 values of cell population below approximately 7.5 mmHg in normal breast and 35 mmHg in patient A breast cancer. There is very little change in the sensitivity of normal breast, and no change in the sensitivity of patient A cell due to FLASH oxygen depletion.

The profile after 20 Gy CONV irradiation is shown in supplementary Figure S3. In the absence of oxygen depletion, the percent of surviving cells following 20 Gy CONV radiation in aggregate breast and patient B breast cancer in the 0-2.5 mmHg bin, is lower than following FLASH irradiation and the overall surviving fraction is reduced.
Figure 3. The pO2 profile of aggregate normal breast, aggregate breast tumor and individual patient breast tumor profiles prior to and following 20 Gy FLASH irradiation. The pO2 frequency distribution prior to irradiation for all cells is indicated by the blue curve, and the profiles of surviving cells following 20 Gy is indicated by the orange curve. The 95% confidence interval of the frequency of cells in each bin is shown.

3.4 Impact of the value of $k$

Figure 4 shows the impact of the value of $k$. With increasing $k$, cells exhibit hypoxic resistance at higher oxygen concentrations independent of dose-rate. In aggregate breast normal tissue, the value of $k$ negligibly impacts the response to FLASH vs. CONV irradiation at doses less than 25 Gy. Again, this is due the relatively high minimum oxygen concentration values observed in normal breast and the low frequency of these lower oxygen concentration values. For aggregate breast cancer, an increase in the value of $k$ is apparent at lower doses due to the relatively hypoxic status of breast cancer. However, the general trend does not apply to well oxygenated breast
cancers such as seen in Figure 3, Patient A. In this case, an increase in $k$ may result in normal tissue protection.

![Figure 4](image.png)

Figure 4. The impact of $k$ on the surviving fractions of (a) normal breast and (b) breast cancer. $g = 0.45 \, \mu M/Gy$ (0.36 mmHg/Gy).

**3.5 Impact of the value of $g$**

Figure 5 shows that the impact of oxygen depletion rate ($g$). Increasing the depletion rate from 0.19 to 0.71 $\mu M/Gy$ does not alter the response to CONV dose-rate irradiation as the rate of oxygen diffusion into cells likely exceeds oxygen depletion. Due to the absence of $pO_2$ values below 12.5 mmHg in normal tissue and the paucity of values in the 12.5-15 mmHg range, the impact of oxygen depletion is not apparent for values of $g$ from 0.19 to 0.71 $\mu M/Gy$ below a dose of 25 Gy. Similarly, the impact of $g = 0.19 \, \mu M/Gy$ increases the dose at which FLASH induced tumor hypoxia is apparent to $>10$ Gy.

One may notice that the SF curves in Figure 4b and 5b are not perfectly smooth. This results from the changing $D_T$ for cells in different $pO_2$ bins. The effect of changing OER and its associated $\alpha$, $\beta$, $\gamma$, and $D_T$ is more pronounced at lower oxygen concentrations. A substantial percent of tumor tissue resides in the low $pO_2$ region and the values in each $pO_2$ bin are very different, resulting in
the wavy curve. This trend is not seen in Figures 4a and 5a as most normal breast tissue exhibits higher oxygen concentrations which do not impact the parameters $\alpha$, $\beta$, $\gamma$, and $D_T$.

Figure 5. The impact of the value of $g$ on the surviving fractions of (a) aggregate normal breast and (b) aggregate breast cancer. $k = 3.8$ mmHg.

3.6 Patient therapeutic benefit

Figure 6 shows SF versus dose curves for aggregate normal breast and breast tumors in panels a and b, and individual patient tumors in panels c and d. The SF curves show that FLASH becomes protective of normal breast tissue due to induced hypoxia starting at doses of approximately 30 Gy. The sparing effect of FLASH induced hypoxia is apparent at a significantly lower dose, i.e., at approximately 10 Gy in aggregate breast tumors. Thus, for the population average pO$_2$ profile, FLASH might be expected to have a negative therapeutic effect relative to CONV irradiation.

However, aggregate cancer patient response does not predict individual patient response. For patient A, FLASH oxygen depletion by a 30 Gy dose is insufficient to reduce any of the measured tumor pO$_2$ values lower than 35 mmHg or impact tumor response. In contrast, FLASH increases tumor hypoxia at doses exceeding 10 Gy in patient B. Although FLASH and CONV dose rate irradiation yield essentially identical survival curves the existence of a small fraction of marginally radiobiologically hypoxic cells in normal tissue can substantially reduce the dose at which a radioprotective effect is observed. This is illustrated by the analysis of the impact of the addition of 1% of cells to the 2.5–5 mmHg pO$_2$ bin to the aggregate normal breast tissue profile,
supplemental Figure S4. In contrast to the impact of 1% cells on the fraction of cells following CONV irradiation, exposure to FLASH irradiation increases the SF by a factor of 25.12 at 20 Gy.

Figure 6 also illustrates the modest effect that the pO₂ frequency uncertainty per bin (95% confidence interval) has on response.

Figure 6. The surviving fractions of aggregate (a) normal and (b) tumor breast tissue, as well as (c-d) patient specific breast tumor following CONV or FLASH irradiation. Calculated with \( g = 0.45 \mu M/Gy \) (0.36 mmHg/Gy) and \( k = 3.8 \) mmHg. The dashed lines indicate the SF calculated with the 95% confidence intervals of pO₂ profiles in Figure 3.

The ratio of the FLASH to the CONV dose to achieve the same surviving fraction (Figure 6) is plotted in Figure 7. For aggregate breast tumors, and patient A and B breast tumors, FLASH hypoxia induction increases the dose of FLASH irradiation to achieve the same SF as 10 Gy CONV irradiation by factors of approximately 1.04, 1.0 and 1.12 respectively.
Figure 7. The ratio of the FLASH dose to the CONV dose to achieve the same surviving fraction is plotted as a function of dose. Panel (a) compares aggregate normal breast to aggregate breast tumor. Panel (b) compares normal breast to individual breast tumors of patient A and B. The upper X-axis indicate the CONV dose to reach the indicated SF in aggregate normal breast.

4 Discussion

The mechanism of the FLASH effect remains unclear, and previous studies indicate that the radical-radical recombination (Labarbe et al., 2020), tissue redox metabolism (Spitz et al., 2019. Montay-Gruel et al., 2019), altered immune/inflammatory response (Eggold et al., 2021; Zhu et al., 2022), and other mechanisms which could play a significant role. Our results show that an evaluation of oxygen depletion as a potential mechanism of tissue sparing by FLASH irradiation requires examination of complete tissue pO2 profiles. Predictions or assessments of response based on tissue median or average pO2 can be significantly misleading. This pertains to CONV and especially FLASH irradiation.

Oxygen is a powerful modifier of cell and tissue response to radiation in the oxygen concentration range of 0-15 mmHg and especially in the 0-7 mmHg range. Our analysis shows that median or average pO2 values do not reveal the fraction or the distribution of cells over that pO2 range. For normal human brain with a median pO2 of 25 mmHg, it may be assumed that it is more poorly oxygenated than human breast cancer which has a median pO2 of 30 mmHg.
However as seen in Figure 2, panels a and c, approximately 30% of breast tumor pO2 values lie in the 0–10 mmHg pO2 range vs. 11% in normal brain. FLASH significantly alters the response of both tissues, a finding not expected based on median tissue pO2 values. Similarly, based on a median pO2 value of 50 mmHg, the predicted response of subcutis to CONV and FLASH irradiation does not differ, but based on analysis of the tissue’s complete profile, FLASH induces substantial radioprotection at doses above approximately 15 Gy.

We examined the impact of different model parameter values on cell response to irradiation. Varying the value of $k$ suggests that for aggregate or average breast tumor and normal tissue, an equal increase in the value of $k$ in both tissues decreases the dose at which hypoxic radioprotection is observed. This is especially true in tumor tissue due to its lower pO2, (Figure 4b versus 4a). The same is true for changing the oxygen depletion rate or $g$ value. An equal increase in $g$ would give rise to protection of both tissues at lower radiation doses (Figure 5). The very limited in vivo data pertaining to FLASH oxygen depletion in tumor versus normal tissue suggest that oxygen depletion in normal tissue may be greater than in tumors. As previously noted, Cao et al. reported that the $g$ value could be more than two times-higher in the normal tissue than in tumor tissue (Cao et al., 2021).

In this study, the pO2 profiles of human normal breast and breast tumor were examined because of the substantial pO2 data available for both tissues. We also included brain and subcutis profiles of pO2 and found that other human tumors and normal tissue pO2 profiles can differ substantially from those observed in breast tissue. Similarly, the pO2 profiles of rodent tumors and various rodent normal tissues may substantially differ from human breast tumor and normal tissue, although such rodent profiles have not been reported. Therefore, it cannot be concluded that the results presented here are in agreement with or differ from studies performed in rodents. However, the principles may be expected to apply to both. For example, for severely hypoxic tumors, as seen in the sarcoma murine tumor (Collingridge et al., 1997), further depleting oxygen by FLASH will not appreciably affect response. Similarly, analyses of normal human brain response to FLASH radiation might be expected to apply to normal rodent brain tissue if the pO2 spectrums are similar. Although not seen in the normal tissue pO2 profiles presented here, the possible presence of a very small population of hypoxic normal tissue stem cells on the threshold of radiobiologic hypoxia (5-
10 mmHg), as previously reported cannot be excluded (Mohyeldin et al., 2010; Pratx and Kapp, 2019a, b). as the Eppendorf system does not resolve pO2 on a cell-by-cell basis. If present, FLASH oxygen depletion would likely contribute to their survival and possibly the repair of radiation injury.

5 Conclusion

This study developed a method for analyzing oxygen depletion during extremely high dose-rate radiation and its consequent effect on cell and tissue response. Our method provides a framework that can be used to estimate which normal tissue and tumor circumstances may benefit from FLASH therapy. The results exposed the inadequacy of using median pO2 for the prediction of tissue response to radiation due to oxygen depletion. Based on the complete pO2 profile of normal human breast and tumor, in the majority of breast cases, CONV irradiation is superior to FLASH irradiation. The results obtained do not pertain to other rodent and human tissues’ response to FLASH irradiation. Aggregate pO2 profiles of normal breast, subcutis and brain reveal significant pO2 profile differences, which will influence oxygen depletion mediated differences in response to FLASH irradiation.

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Supplementary Material

1. Summary of previously reported values of $g$

Weiss et al. measured the value of $g$ by irradiating an oxygen-equilibrated bacterial cell suspension in a sealed vessel and reported $g = 0.58 \pm 0.1 \, \mu\text{M/Gy}$ under conventional dose rate and $g = 0.26 \pm 0.05 \, \mu\text{M/Gy}$ at ultra-high dose rate thin layer conditions i.e. bacteria coated with a film of culture medium (Weiss et al., 1974). Michaels measured the value of $g$ in stirred aqueous solutions of CHO cells in sealed glass vessels and reported $g = 0.44 \, \mu\text{M/Gy}$ (Michaels, 1986), similar to the value of $g$ evaluated in a thin layer technique $g = 0.48 \, \mu\text{M/Gy}$ (Michaels et al., 1978). Epp et al. reported $g = 0.61 – 0.71 \, \mu\text{M/Gy}$ in HeLa cells (Epp et al., 1972). Nias et al. reported $g = 0.65 \, \mu\text{M/Gy}$ in HeLa cells using 1 μs pulsed electrons (Nias et al., 1969). Boscolo et al. reported $g = 0.33 \, \mu\text{M/Gy}$ for 1 MeV electrons by Monte Carlo (MC) simulation (Boscolo et al., 2021). Lai et al. reported $g = 0.19 – 0.22 \, \mu\text{M/Gy}$ for 4.5 keV electrons at a dose rate of $10^6 – 10^8 \, \text{Gy/s}$ by MC simulation (Lai et al., 2020), and Zhu et al. obtained $g = 0.38 – 0.43 \, \mu\text{M/Gy}$ for 4.5 MeV electrons with MC simulation (Zhu et al., 2021).
2. Reproducing experimental data (HeLa cells)

The following figure shows the SF curves of HeLa cells obtained by Epp et al. (Epp et al., 1972) and reproduced with the method described in section 2.2 of the manuscript. It should be noted that the original data extracted from Epp et al. were uncorrected for cellular multiplicity ($N$), which was estimated average $N = 2$. Cellular multiplicity refers to the number of cells per colony forming unit. The determination of SF curve inactivation parameters requires single cell suspensions with $N = 1$. Assuming the survival probability of a colony forming unit after a dose of $D$ is $m$, then the survival probability of a cell in a colony forming unit with a cellular multiplicity of $N$ is $SF = 1 - (1-m)^{1/N}$. If uncorrected $N$ higher than 1 results in shallower curve slope and incorrect SF shape. To correct for the cellular multiplicity effect, the experimental data from Epp et al. were corrected with $SF_{corrected} = 1 - \sqrt{1 - SF_{Epp}}$ where $SF_{Epp} = m$. More comprehensive corrections require knowledge of the discrete multiplicity (not just the average multiplicity, and the value of “m” at zero dose (Gerweck et al., 1994). These data were not provided.

After data correction, the $\alpha$ and $\beta$ values were derived from the $N_2$ curve with $D_T = 12$ Gy and used to reproduce the SF curve of other oxygen concentrations (with OER adjustments of $D_T$). The following figure shows that our reproduced dashed lines fit experimental data in the high dose region. Data and fits to two additional oxygen concentrations < 0.91% O$_2$, is not plotted for clarity. In general, there was substantial scatter in the experimental data at SF values > 0.1 but the agreement between the predicted and observed SF values was strong at SF < 0.1. Figure 1 of the manuscript shows fits of additional data sets.

Figure S1. The SF curves of HeLa cells exposed to simulated FLASH irradiation along with the experimental data of Epp et al. (Epp et al., 1972). $\alpha_{anoxic} = 0.09097$, $\beta_{anoxic} = 0.003867$, $D_T = 12$ Gy, $g = 0.368$ mmHg/Gy.
Figure S2. Graphic description of analysis method based on the full pO2 profile. ① shift the pO2 profile by $g \times$ fractional dose; ② calculate the surviving fraction in each bin using the adjusted $\alpha$, $\beta$, $\gamma$, $D_T$ values; ③ repeat the process until a preset total dose is delivered; ④ normalize the pO2 profile so that the sum of frequencies in all bins equals 100%.
Figure S3. The $pO_2$ profile of (a) aggregate normal breast; (b) aggregate breast cancer; and (c-d) patient specific breast tumors before (blue lines). The error bars in panels a - d are 95% confidence intervals. Calculated with $g = 0.45 \mu M/Gy (0.36 \text{ mmHg/Gy})$. 
Figure S4. The impact of 1% cells on the threshold of hypoxia. (a) The pO2 profile of aggregate normal breast with 1% tissue in the 2.5-5 mmHg bin. (b) The SF curves of original aggregate normal breast (black lines) and aggregate normal breast with 1% tissue in the 2.5-5 mmHg bin (red lines).

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