FLASH Depletion of Oxygen in Tumor and Normal Tissue, and its Likely Consequences

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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, and the oxygen concentration yielding half-maximal radiosensitization in tumor and normal tissue. Previously reported ranges and uncertainties in the aforementioned parameters were used to calculate the response of normal breast and breast tumor based on the linear quadratic-linear (LQ-L) model. The results suggest that the therapeutic efficacy of FLASH oxygen depletion is likely patient dependent and beneficial in a minority of cases. Circumstances under which FLASH oxygen depletion could be of therapeutic benefit or deficit were identified.

1 Introduction

In 2014 it was reported that a single dose of 20 Gy radiation with electrons administered to the thorax of rats at a mean dose rate ≥ 40 Gy/s (FLASH irradiation) resulted in “no lung complications”, whereas 15 Gy administered at a conventional (CONV) dose rate (≤ 0.03 Gy/s) lead to significant lung fibrosis (Favaudon et al., 2014). Additionally, FLASH and CONV
irradiation equally repressed 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, the mechanism(s) of tissue sparing have not yet been resolved (Adrian et al., 2020; Wardman, 2020; Pratx and Kapp, 2019; Spitz et al., 2019; Wilson et al., 2020; Jin et al., 2020; Petersson et al., 2020; Labarbe et al., 2020). Additionally, most but not all studies have reported normal tissue sparing at FLASH dose rates (Venkatesulu et al., 2019; Smyth et al., 2018).

It has long been known that both CONV and FLASH irradiation deplete dissolved oxygen in aqueous solutions. The 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 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.

As pertains in vitro, the extent to which FLASH oxygen depletion impacts tissue response will depend on pretreatment tissue $pO_2$, oxygen depletion per unit dose, total dose and the oxygen concentration at which half-maximal sensitization occurs. In this study, we examined 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.:

\[
\alpha_{aerobic} = \alpha_{anoxic} \times \text{OER} \tag{2}
\]

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

\[
\text{OER} = \frac{k + m \times [O_2]}{k + [O_2]} \tag{4}
\]

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:

\[
D_T|_{aerobic} = D_T|_{anoxic} / \text{OER} \tag{5}
\]

### 2.2 Reproducing experimental data and testing the model

The SF curves obtained by Ling et al. (Ling et al., 1978) were reproduced to test and verify our method. In the experiments performed by Ling et al, Chinese hamster ovary (CHO) cells with different oxygen concentrations (0.21% and 0.44%) were exposed to two pulses of electrons, each
lasting 3 ns. The first pulse with a dose of $D_1 = 12$ Gy was delivered to deplete intracellular oxygen and the second pulse with a variable dose $D_2$ was delivered with a time delay of 10 ns or 60 s. Experimental data reported by Ling et al. and others, e.g. (Weiss et al., 1974; Epp et al., 1972) show that the oxygen depletion and radiation damage which leads to cell death occur within the 3 ns dose duration. To quantify the impact of oxygen depletion during the 3 ns pulse duration on the radiation sensitivity parameters $\alpha$, $\beta$ and $\gamma$, we utilized the Alper and Howard-Flanders competition model (Alper and Howard-Flanders, 1956) and a micro pulse size of 1 Gy with 0 time elapsing between each micro pulse. Practically, this was implemented via the following steps:

1. Deliver the $n^{th}$ micro-pulse 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 $n^{th}$ micro-pulse is then calculated in a recursive manner for each micro-pulse: $SF(n \text{ Gy}) = SF(n - 1 \text{ Gy}) \times (1 - d_{SF|n})$

2.3 Application of the model to human tissue

A treatment site (breast) for which substantial pO$_2$ data is available (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. obtained aggregate normal breast pO$_2$ profiles of $N=16$ patients, $n=1009$ evaluated foci along with breast tumor pO$_2$ profile 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 were extracted from (Vaupel et al., 1991) 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 exceeds the rate of oxygen depletion and the tissue pO$_2$ profile was unchanged.
2. For FLASH irradiation, the pO$_2$ profile was shifted by $g$ after each 1 Gy micro-pulse; the parameters OER, and OER adjusted $\alpha$, $\beta$, $\gamma$, $D_T$ values were updated in each bin to calculate the SF using the micro-pulse 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. 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 N$_2$ SF curve in Figure 1a with the LQ-L model. $g = 0.275$ µM/Gy was adopted as their data indicate 12 Gy depletes 0.44% oxygen (3.3 mmHg). 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.

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. Weiss et al. measured the value of $g$ by irradiating an oxygen-equilibrated bacterial cell suspension in a sealed vessel and reported $g = 0.58 \pm 0.1$ µM/Gy under conventional dose rate and $g = 0.26 \pm 0.05$ µM/Gy at ultra-high dose rate under 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 contained 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 using 3 ns pulsed electrons (Epp et al., 1972). Nias et al. reported \( g = 0.65 \, \mu \text{M/Gy} \) in HeLa cells using 1 \( \mu \text{s} \) 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). In this work we adopted the minimum and maximum reported value of \( g \), i.e., \( g = 0.19 \) and 0.71 \( \mu \text{M/Gy} \) (0.15 and 0.56 mmHg/Gy) to investigate the impact of the value of \( g \) uncertainty on cell survival. We further used the mean reported \( g \) (0.45 \( \mu \text{M/Gy} \), i.e., 0.36 mmHg/Gy) as the FLASH oxygen depletion rate for other calculations.

3 Results

Figure 1 shows the SF curves of CHO cells exposed to simulated FLASH electron micro-pulses with a time interval of 0 s between pulses along with the experimental data of Ling et al (Ling et al., 1978). As seen, the calculated SF curves based on the \( \alpha \) and \( \beta \) values derived from the \( \text{N}_2 \) SF curve in Figure 1a described the final anoxic slope correctly and showed satisfactory agreement with the measured data. One may note that the \( \text{N}_2 \) SF curves in Figure 1a and Figure 1b are slightly different, due to experimental uncertainties, and an equally good fit can be achieved with the \( \alpha \) and \( \beta \) values derived from the \( \text{N}_2 \) SF curve in Figure 1b and \( D_T = 10 \, \text{Gy} \) (see Figure S1).
Figure 1. The fraction of Chinese hamster ovary (CHO) cells surviving 3 ns electron FLASH irradiation under different initial oxygen concentrations. The survival of cells equilibrated with nitrogen is indicated by black dots; 0.22% and 0.44% oxygen by blue and red triangles. Original data is extracted from Ling et al. Open yellow circles represent the calculated results obtained with the micro-pulse method. $\alpha_{\text{anoxic}} = 0.0156$, $\beta_{\text{anoxic}} = 0.0071$ and $D_T = 12$ Gy.

Figure 2 shows the pO$_2$ profile of normal breast and breast tumor before and after 20 Gy FLASH irradiation. The final relative frequency of tissue in each 2.5 mmHg bin were obtained by following 3 steps after each 1 Gy micro-pulse: ① shift the pO$_2$ profile by $g$; ② calculate the surviving fraction in each bin using the adjusted $\alpha$, $\beta$, $\gamma$, $D_T$ values; ③ normalize the pO$_2$ profile so that the sum of frequencies in all bins equals 100%. These steps are illustrated for aggregate normal breast as an example to present the pO$_2$ profile appearance after a 20 Gy dose delivery. (Figure 2, panel a.1-a.3).

Figure 2b shows the pO$_2$ profile of aggregate breast tumor cells prior to irradiation (black line); the pO$_2$ profile of cells that survive 20 Gy FLASH irradiation is indicated by the red line. Aggregate breast tumor tissue is substantially more hypoxic than normal breast prior to irradiation, with approximately 15% of pO$_2$ values being < 5 mmHg, and 30% < 10 mmHg. 20 Gy FLASH irradiation further reduces the pO$_2$ by approximately 7.2 mmHg and nearly 100% of cells surviving 20 Gy reside in the 0-2.5 mmHg bin.
Figure 2 panels c and d reveal the substantial intertumoral pO$_2$ heterogeneity observed from patient to patient and its likely impact on patient response to radiation. For these individual tumors in the cohort of 15 tumors, 20 Gy FLASH reduces the mean pO$_2$ by approximately 7.2 mmHg, however none of the tumor foci of patient A (panel c) exhibits radiobiologic hypoxia. In contrast, in patient B (panel d), approximately 16% of all foci are between 0 and 2.5 mmHg, and approximately 50% of foci pO$_2$ values are < 5 mmHg prior to irradiation. Following 20 Gy irradiation, greater than 99% of all surviving cells reside in the 0-2.5 mmHg pO$_2$ bin category.

As the normal breast pO$_2$ profiles of patients A and B were not shown in Vaupel et al., and patient normal breast tissue pO$_2$ has not been reported to predict patient tumor pO$_2$, the tumor pO$_2$ profile of patient A and B was compared to the aggregate normal breast pO$_2$ profile, as shown in Figure 3.

![Figure 2](image)

Figure 2. The pO2 profile of aggregate normal breast (a.1-a.3), aggregate breast tumor (b) and patient specific breast tumors (c-d) before (black solid line) and after 20 Gy FLASH irradiation (red dashed line). $g = 0.45 \text{ μM/Gy} (0.36 \text{ mmHg/Gy})$ was used in the calculation. Panels a.1 and a.2 show the steps to obtain the pO2 profile after FLASH, which includes shifting the pO2 profile 20 times by $g$ after each micro-pulse (a.1) and calculation of the surviving fraction in each bin (a.2); Panels a.3 and b-d present the final pO2 profiles after normalization such that the sum of all bins equals 100%.
Figure 3 shows the SF curves versus dose for aggregate normal breast and breast tumors in panels a and b, and individual patient tumors in panels c and d. FLASH is increasingly protective of normal breast tissue due to induced hypoxia with increasing dose 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 patient response does not predict individual patient response. For patient A, FLASH oxygen depletion by a 30 Gy dose is insufficient to render 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.

Figure 3. 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 dose equivalence of the SF differences between FLASH and CONV irradiation due to FLASH oxygen depletion, was used to calculate the ratio of the FLASH to CONV dose to yield the same SFs. This ratio is referred to as RBE$_{FLASH}$ in this work. The results are plotted as a function of SF in Figure 4. For the aggregate population of 15 tumors, the fraction of FLASH irradiated tumor tissue exhibiting RBE$_{FLASH}$ increases with dose to a value of 1.3 at a FLASH dose of approximately 25 Gy (panel a). The RBE$_{FLASH}$ of aggregate normal breast increased much slower than for aggregate tumor, as a much larger dose is required to shift the pO$_2$ profile to the hypoxic region.
For patient A and B tumors, a contrasting FLASH effect is observed. For the well oxygenated tumor of patient A (blue curve), the killing efficiency of FLASH and CONV are virtually identical for doses up to 30 Gy, as 30 Gy FLASH is insufficient to induce significant radiobiologic hypoxia. However, for the relatively hypoxic tumor of patient B, FLASH is significantly less effective (more tumor sparing) than CONV radiation. Thus, considering the effect of FLASH versus CONV irradiation in both tumors and aggregate normal breast tissue (red curve in Figure 4b), FLASH provides a small advantage vs. CONV irradiation, due to the induction of hypoxia in normal tissue beginning at a high dose of approximately 30 Gy. In contrast, for patient B harboring a relatively hypoxic tumor, with more than 30% of pO$_2$ values in the 2.5-5.0 mmHg range, FLASH induced hypoxic resistance increases rapidly with dose and CONV irradiation is superior at all doses up to 50 Gy. For aggregate breast tumors, and patient A and B breast tumors, hypoxia induction by 10 Gy FLASH increases the dose to achieve the same effect in tumor vs. normal breast tissue by factors of approximately 1.15, 1.0 and 1.22, respectively.

Figure 4. RBE$_{\text{FLASH}}$, which is calculated as the ratio of the FLASH dose to the CONV dose to reach the same SF, in normal breast tissue and (a) breast tumor and (b) patient specific breast tumors. Calculated with $g = 0.45$ μM/Gy (0.36 mmHg/Gy) and $k = 3.8$ mmHg. Triangles mark RBE$_{\text{FLASH}}$ values of 10 Gy.

Figure 5 shows the impact of the value of $k$. With increasing $k$, cells exhibit hypoxic resistance at higher oxygen concentrations independent of dose-rate, although the effect is less pronounced in aggregate normal breast tissue due to its relatively high initial oxygen concentration. 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 to the relatively high minimum oxygen concentration values observed in normal breast and the low frequency of these lower oxygen concentration values. In general, for aggregate breast normal and tumor tissue, an increase in the value of $k$ is more protective of tumor than normal tissue due to the lower $pO_2$ values and a higher frequency of lower $pO_2$ values in tumors as seen in Figure 1a, vs. Figure 1b. However, this does not pertain in all cases as seen in Figure 1c, for which an increase in $k$ would be expected to protect normal vs. tumor tissue.

One may notice that the SF curves in Figure 5b and 6b 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 5a and 6a as most normal breasts are at higher oxygen concentrations and the parameters in each bin do not enter the low oxygen concentration necessary to impact the parameters $\alpha$, $\beta$, $\gamma$, and $D_T$.

Figure 5. The impact of $k$ on the surviving fractions on (a) normal breast and (b) breast cancer. $g = 0.45 \mu M/Gy (0.36 \text{ mmHg/Gy})$.

Figure 6 shows that the impact of increasing the oxygen depletion rate ($g$) from 0.19 to 0.71 $\mu M/Gy$ does not alter the response to CONV dose-rate irradiation since the rate of oxygen diffusion into
cells likely exceeds oxygen depletion. However, at extremely high dose-rates, when oxygen depletion exceeds resupply, significant resistance develops during irradiation (Ling et al., 1978; Weiss et al., 1974; Epp et al., 1972; Nias et al., 1969). As seen in Figure 6a, 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 $g$ below a dose of 25 Gy. At the lowest consumption rate, 0.19 μM/Gy, the dose response curve is marginally affected. This contrast with tumor response, for which the impact of a $g = 0.1$ μM/Gy oxygen depletion rate is apparent at 10 Gy. This is due to the fact that tumors contain a relatively large fraction of cells which are on the threshold of radiobiologic hypoxia, i.e., 5-7 mmHg O$_2$.

![Figure 6. The impact of the value of $g$ on the surviving fractions of (a) normal breast and (b) breast cancer. $k = 3.8$ mmHg.](image)

**4 Discussion**

The present study investigates the dynamics of oxygen depletion as a potential mechanism of FLASH tissue sparing. Our model examines the impact of oxygen depletion during ultra-high dose-rate, i.e., FLASH irradiation, on radiation sensitivity. This model was used to calculate surviving fractions for a range of parameters impacting pO$_2$ in irradiated tissue based on the oxygen concentration profiles of breast cancer patients. The results indicate that the potential impact of FLASH oxygen depletion on therapeutic response is dependent on the pO$_2$ profiles of both tumor and normal tissue.
For pO2 profiles reported for breast tumor and normal tissue, radiobiologic hypoxia is unlikely to be induced in normal breast tissue at doses less than 25-30 Gy. In contrast, in the majority of tumors, FLASH oxygen depletion is likely to modestly increase resistance following single acute doses exceeding 5-10 Gy. There are exceptions however. The pO2 profile of human breast tumors is somewhat bimodal, with half of all measured foci exhibiting pO2 values of greater than 20 mmHg. In the subgroup of tumors exhibiting high pO2 values, i.e., greater than 20 mmHg in most or all foci, as seen in patient A, Figure 3c, induced hypoxia is not observed at doses below 30 Gy. For normal breast tissue, a radioprotective hypoxic effect is predicted due to FLASH oxygen depletion for doses above 30 Gy.

The influence of varying g and k values equally in both tumor and normal tissue does not alter the oxygen depletion trends in either tissue, but rather the dose at which they become apparent. If the values of g or k differ between tumor and normal tissue e.g., oxygen depletion being more prominent in normal than tumor tissue, then oxygen depletion could differentially impact tissue response and the therapeutic ratio. Such a possibility is suggested by the recent study of Cao et al (Cao et al., 2021), who utilized oxygen dependent phosphorescence quenching of the molecular probe Oxyphor 2P to evaluate in vitro and in vivo extracellular FLASH oxygen depletion. FLASH oxygen depletion in subcutaneous tumors in mice was approximately 50% of that observed in normal subcutaneous tissue. Although the authors noted the relatively low rate of oxygen depletion in tumor may have been due to the presence of necrotic areas within the sampled volume, this and related studies warrant substantial additional investigation. If confirmed, a factor of 2 greater oxygen depletion rate in normal vs. tumor tissue could result in significant normal tissue protection.

Very few in vivo studies yield data pertaining to the value of k. An exception is the study by (Collingridge et al., 1997). These investigators evaluated the pO2 profile of a murine tumor with the polarographic Eppendorf system and a fiberoptic luminescence sensor. The percent oxygen partial pressure values <10, <5, and <2.5 mmHg were 89%, 79%, and 69% in the polarographic probe analysis and 83%, 75% and 50% as analyzed by the luminescence probe, respectively. Both data sets flank and approximate the oxygen concentration which yields half-maximum radiation sensitization in vitro, i.e., 3-5 mmHg, and is similar to the 67% radiobiologically determined
hypoxic cell fraction of the tumor. These results suggest that the value of $k$ does not significantly differ in vitro and in vivo.

Kahn et al. (Khan et al., 2021) evaluated the impact of FLASH irradiation on the survival of cells in a tumor spheroid model. As observed in vitro, at sufficiently high doses, cell killing per unit dose decreased. Cell survival was consistent with a model using an oxygen depletion value of 1.8 mmHg/Gy and a 1.9 mmHg $k$ value. Although the value of $g$ was substantially higher and the $k$ value was substantially lower than observed by others in vitro and in vivo (Epp et al., 1972; Nias et al., 1969; Ling et al., 1978; Michaels et al., 1978), the impact of the relatively extreme values may have been minimized by the contrasting impact of each on the dose needed to induce hypoxic resistance.

The results of this study cannot be extrapolated to all tumors and associated normal tissues. For example, in a two patient study by Kallinowski et al, no pO$_2$ values <5 mmHg were observed in normal cervix tissue but approximately 7% of values ranged between 5 and 10 mmHg (Kallinowski et al., 1990). Oxygen depletion by a FLASH dose of 10-20 Gy may be expected to induce significant hypoxia dependent radioprotection of normal cervical tissue in contrast to conventional irradiation.

Although not seen in the normal tissue pO$_2$ profiles in the present study (Figure 2a), reports suggest that normal tissue stem cells exhibit characteristics which suggest they exist in a low oxygen environment, i.e., a “hypoxic stem cell niche” consisting of 1% or fewer normal tissue cells, e.g., (Mohyeldin et al., 2010; Pratx and Kapp, 2019). It is unlikely that such niches can be directly resolved with clinically useful oxygen probes such as the Eppendorf system or other non-invasive or minimally invasive probes, especially if dispersed within the normal tissue parenchyma. However, the existence of a small fraction of marginally radiobiologically hypoxic cells in normal tissue may be expected to significantly alter the biological response to FLASH vs. conventional dose-rate irradiation. As shown in supplementary Figure S2, a 1% presence of normal tissue cells in the 5-7.5 mmHg bin or the 7.5-10 mmHg bin markedly increases the percentage of cells surviving FLASH irradiation (panel a and b) and substantially reduces the dose at which a radioprotective effect is observed (panel c). The development of methods to identify such hypoxic
niches and their role in normal tissue radiation damage repair are needed to more fully resolve and exploit the role of oxygen depletion as a mechanism of normal tissue protection.

Our analysis presents a method to estimate the FLASH effect based on oxygen depletion using oxygen profiles obtained from patient tissues. The oxygen depletion model is consistent with experimental in vitro data (Nias et al., 1969; Epp et al., 1972; Ling et al., 1978; Weiss et al., 1974). Multiple studies have shown that rodent tumor models can be treated with FLASH doses up to 20 Gy without loss of efficacy, as well as a radioprotective effect of FLASH on normal tissue (Kim et al., 2021; Diffenderfer et al., 2020; Montay-Gruel et al., 2020; Favaudon et al., 2014). The minimum and maximum FLASH dose to achieve normal tissue protection may be tissue or organ dependent and remains under investigation (Montay-Gruel et al., 2019; Gao et al., 2021; Fouillade et al., 2020; Cunningham et al., 2021; Zhang et al., 2020). These data would suggest that additional effects such as a hypoxic normal tissue stem cell niche, differential depletion of oxygen between tumor and normal tissue as reported in recent studies, or other additional mechanisms may contribute to a FLASH effect.

5 Conclusion

Based on polarographic electrode estimates of the oxygen status of patient breast tumor and normal tissue, for similar radiolytic oxygen depletion per unit dose in both tissues, our model predicts that FLASH oxygen depletion increases tumor hypoxia and radiation resistance but is without significant effect on the radiation sensitivity of normal tissue in the majority of cases. In a subset of well oxygenated tumors, at high doses a therapeutic gain may be realized due to the induction of normal tissue hypoxia. If normal tissues contain a small fraction of stem cells in a low pO2 environment, that is, a stem cell niche, FLASH oxygen depletion could be of substantial therapeutic benefit. Additionally, if oxygen depletion per unit dose is greater in normal than in tumor tissue as recently reported, our analysis again suggests FLASH irradiation could be of therapeutic benefit. These reports warrant further investigation and validation.
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Figure S1 shows the SF curves of CHO cells exposed to simulated FLASH electron micro-pulses with a time interval of 0 s between pulses along with the experimental data of Ling et al. Here, the calculated SF curves were based on the $\alpha$ and $\beta$ values derived from the N$_2$ SF curve in Figure S1b.

Figure S1. The fraction of Chinese hamster ovary (CHO) cells surviving 3 ns electron FLASH irradiation under different initial oxygen concentrations. The survival of cells equilibrated with nitrogen is indicated by black dots; 0.22% and 0.44% oxygen by blue and red triangles. Original data is extracted from Ling et al. Open yellow circles represent the calculated results obtained with the micro-pulse method. $\alpha_{anoxic} = 0.0156$, $\beta_{anoxic} = 0.0083$ and $D_T = 10$ Gy.
Figure S2 illustrates the impact of a small percent of normal tissue cells residing in a low pO$_2$ niche of the aggregate breast normal tissue as seen in Figure 1. In panel a, 1% of cells reside in the 5.0-7.5 mmHg pO$_2$ bin; in panel b they are found in the 7.5-10 mmHg bin. Black lines indicate the percent of all cells in each bin prior to irradiation; red lines indicate the percent surviving cells in each bin following 20 Gy irradiation. Panel c shows the impact of the 1% of cells in the 5 mm-7.5 mmHg pO$_2$ bin, or 7.5-10 mmHg bin, on the dose-response survival curves. FLASH drives these threshold hypoxic cells deeper into hypoxia, with an associated increase in radioresistance.

Figure S2. The tested pO$_2$ profile with 1% cells added to the 5-7.5 mmHg bin (a) or 7.5-10 mmHg bin (b) in the aggregate normal breast pO$_2$ profile, and the corresponding surviving fractions (c).