Modeling of cellular response after FLASH irradiation: a quantitative analysis based on the radiolytic oxygen depletion hypothesis

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Abstract

Purpose. Recent studies suggest ultra-high dose rate (FLASH) irradiation can spare normal tissues from radiotoxicity, while efficiently controlling the tumor, and this is known as the 'FLASH effect'. This study performed theoretical analyses about the impact of radiolytic oxygen depletion (ROD) on the cellular responses after FLASH irradiation. Methods. Monte Carlo simulation was used to model the ROD process, determine the DNA damage, and calculate the amount of oxygen depleted (\(L_{ROD}\)) during FLASH exposure. A mathematical model was applied to analyze oxygen tension (\(pO_2\)) distribution in human tissues and the recovery of \(pO_2\) after FLASH irradiation. DNA damage and cell survival fractions (SFs) after FLASH irradiation were calculated. The impact of initial cellular \(pO_2\), FLASH pulse number, pulse interval, and radiation quality of the source particles on ROD and subsequent cellular responses were systematically evaluated. Results. The simulated electron \(L_{ROD}\) range was 0.38–0.43 \(\mu\)M Gy\(^{-1}\) when \(pO_2\) ranged from 7.5 to 160 mmHg. The calculated DNA damage and SFs show that the radioprotective effect is only evident in cells with a low \(pO_2\). Different irradiation setups alter the cellular responses by modifying the \(pO_2\). Single pulse delivery or multi-pulse delivery with pulse intervals shorter than 10–50 ms resulted in fewer DNA damages and higher SFs. Source particles with a low linear energy transfer (LET) have a higher capacity to deplete oxygen, and thus, lead to a more conspicuous radioprotective effect. Conclusions. A systematic analysis of the cellular response following FLASH irradiation was performed to provided suggestions for future FLASH applications. The FLASH radioprotective effect due to ROD may only be observed in cells with a low \(pO_2\). Single pulse delivery or multi-pulse delivery with short pulse intervals are suggested for FLASH irradiation to avoid oxygen tension recovery during pulse intervals. Source particles with low LET are preferred for their conspicuous radioprotective effects.

1. Introduction

Ultra-high dose rate (FLASH) irradiation has attracted attention in the field of medical physics after promising results of several in vivo experiments (Favaudon et al. 2014, Montay-Gruel et al. 2019, Vozenin et al. 2019a) reported in recent years, which have shown that FLASH can spare healthy tissues, while efficiently controlling tumor, a phenomena referred to as the FLASH effect (Vozenin et al. 2019b, Wilson et al. 2020). FLASH irradiation refers generally to have a mean dose rate of \(>40\) Gy s\(^{-1}\) and instantaneous dose rate of \(>10^3\) Gy s\(^{-1}\), and this is of 3–4 magnitude higher than conventional dose rate (CONV) irradiation adopted in clinical applications. Although the underlying mechanism of the FLASH effect remains unclear, the successful treatment of the first
cancer patient using FLASH radiotherapy (Bourhis et al 2019) makes it a promising technique for clinical application.

Several non-mutually exclusive hypotheses have been proposed to explain the FLASH effect, including those based on the modified transforming growth factor beta signaling (Favaudon et al 2014, Buonanno et al 2019), a reduced dose of immune cells in the circulating blood system (Durante et al 2018, Jin et al 2020), and radiolytic oxygen depletion (ROD) (Montay-Gruel et al 2019, Spitz et al 2019, Pratx and Kapp 2019a) during FLASH irradiation. The ROD hypothesis, the leading hypothesis to explain FLASH effect, was first proposed to explain the in vitro experimental results obtained in the earlier studies (Town 1967, Berry et al 1969). When irradiated with a single pulse beam that lasted for nanoseconds (ns) to microseconds (μs), cells were found to be more radioresistant than when exposed to CONV irradiation (e.g. one administered using 60Co) (Berry et al 1969). These results were explained by the rapid depletion of oxygen during FLASH irradiation, which is not possible under CONV irradiation, and by creating a local radiobiological hypoxic environment, thus making cells less radiosensitive. Some other studies reported that FLASH pulses induce a reduced radiosensitivity only in cells with a low level of oxygen (Berry and Stedeford 1972, Adrian et al 2020). More recently, Montay-Gruel et al found the neurocognitive benefits of FLASH irradiation in mice whole-brain irradiation experiments and the benefits were reversed when the oxygen concentration (OC) was doubled in the brain during FLASH irradiation. These results were explained by the hypothesis that the yield of reactive oxygen species (ROS) was reduced due to the rapid ROD in the brain during FLASH irradiation (Montay-Gruel et al 2019).

Oxygen is regarded as one of the key factors that modifies radiobiological responses, and experimental results have shown that hypoxic cells are more radioresistant than their aerobic counterparts (Hall and Giaccia 2018). Oxygen-dependent radiosensitivity is usually explained by the oxygen fixation hypothesis (OFH), which considers that ionizing radiation can produce DNA radicals via chemical reactions, DNA radicals can be chemically restored by thiols in the cellular environment or react with dissolved oxygen molecules and form DNA peroxyl radicals, which are difficult to be repaired and can be regarded as fixed damage. Therefore, hypoxic cells suffer less DNA damage after irradiation (Liu et al 2015, Hall and Giaccia 2018).

Monte Carlo track structure (MCTS) codes can simulate the detailed physics tracks and chemical reactions of primary particles at the nanometer (nm) scale, and have been extensively applied in cellular and sub-cellular radiobiological response modeling under CONV irradiation (Meylan et al 2017, Friedland et al 2017, Zhu et al 2020). Current MCTS codes simulate particle tracks independently, which is realistic under low dose rate irradiation; however, incident particles are densely distributed in spatial and temporal proximity under FLASH irradiation, and intertrack reactions should be considered in FLASH simulations and such simulation can be very time consuming. Some recent progresses were made to adapt MCTS codes for FLASH simulation. Ramos-Méndez et al incorporated the independent reaction time method in the TOPAS-nBio code to study the intertrack effects in the chemical stage of water radiolysis under FLASH irradiation (Ramos-Méndez et al 2020). Lai et al implemented oxygen related chemical reactions in the graphical processing unit-based GMicroMC code and applied it for the study of ROD hypothesis of FLASH irradiation, the oxygen enhancement ratio was calculated to quantify changes in biological effects due to changes in OC (Lai et al 2020). Further study of the FLASH induced chemical reactions and cellular responses should be performed.

In this study, the impact of ROD on cellular responses after FLASH irradiation was quantitatively analyzed using Monte Carlo (MC) simulations and mathematical modeling. The MCTS code NASIC (Nanodosimetry Monte Carlo Simulation Code) was adapted to the FLASH simulations, and the oxygen depletion process and information related to DNA damage under different irradiation setups were obtained. The recovery of oxygen tension (pO2, in the unit of mmHg) in cells due to oxygen diffusion from vessels was described using differential equations and solved using a numerical method. The impact of initial oxygen tension, FLASH pulse structure, and radiation quality on the cellular responses were systematically analyzed in this work.

2. Methods and materials

2.1. MC simulation of FLASH pulse

Currently, almost all published MCTS simulation studies of radiobiological responses have been performed using the CONV irradiation setup and adopted the assumption that particle tracks are independent of each other (Meylan et al 2017, Friedland et al 2017, Zhu et al 2020). Simulations of the physical stage (< 10−15 s), the pre-chemical stage (10−15–10−12 s), and the chemical stage (10−12–10−6 s) were performed sequentially for each primary particle. For the modeling of DNA damage, the chemical stage was terminated within a few nanoseconds (Nikjoo et al 2001, Friedland et al 2003, Friedland et al 2017, Meylan et al 2017, Zhu et al 2020) because it is assumed that the indirect DNA damage results from interactions between the DNA structure and the hydroxyl radicals (OH), which have a lifetime of a few nanoseconds in the cellular environment (Roots and Okada 1975).
Such a simulation method cannot be applied to FLASH simulation owing to two main differences between FLASH irradiation and CONV irradiation. First, a tremendously high number of particles are emitted in a single short pulse (ns to μs scale) and the particle tracks are highly overlapped. Though the intertrack physical reactions can be neglected (Kreipl et al 2009, Ramos-Mendez et al 2020) because the temporal separation between the particle tracks is much greater than the duration of the physical stage (Ramos-Mendez et al 2020), the track overlap can have a significant impact on the chemical reactions because chemical radicals generated by different primary particles can react among themselves. Kreipl et al investigated the impact of spatial and temporal track overlap on the chemical radical yields, and their results showed that the radical yields were considerably modified by intertrack reactions (Kreipl et al 2009).

Second, \( e_{aq} \) and H radicals, which are the main \( O_2 \) scavengers, are produced in response to the FLASH pulse at very high instantaneous concentrations; \( O_2 \) is rapidly depleted during FLASH irradiation due to the chemical reactions of equations (1)–(2) (Nikjoo et al 2006, Boscolo et al 2020, Spitz et al 2019), leading to a significant change in the oxygen tension in the biological tissues, radical reactions and time evolution (Lai et al 2020, Boscolo et al 2021), followed by modified radiobiological response. Therefore, the change in \( O_C \) should be carefully recorded during FLASH irradiation to model the biological response, and the chemical stage should be tracked for the entire lifetime of \( e_{aq} \) and H. Roots et al measured the lifetime of \( OH, e_{aq} \) and H in the cellular environment, which was reported to be 1.6–4.4 ns, 0.19–4 μs, and 40–170 μs, respectively (Roots and Okada 1975)

\[
\begin{align*}
\cdot H + O_2 & \rightarrow HO_2 \\
e_{aq} + O_2 & \rightarrow O_2^-. 
\end{align*}
\]

In this study, the MCTS code NASIC (Li et al 2015, Chen et al 2017) was used for MC simulations of FLASH irradiation. NASIC has already been applied to simulations of radiobiological responses, including DNA damage and the radiosensitization effect of gold nanoparticles under CONV irradiation (Xie et al 2013, Li et al 2015, Chen et al 2017, Li et al 2020); NASIC was further modified for simulations under FLASH irradiation.

In the physics stage, the track structure of \( N \) primary particles that can transverse the cell nucleus during a FLASH pulse were simulated independently, and all the energy depositions induced by primary particles and their secondary particles were recorded as a track structure file. This file was used as the input file for the simulation of the pre-chemical stage to generate sub-excited electrons, excited water molecules, and ionized water molecules, and simulate the following radiolysis process. In this manner, the initial chemical radicals produced by different primary particles in the pulse could be considered in the same pool, and thus, their interactions with each other could be simulated.

The chemistry module of NASIC was modified to support the simulation in oxygenated water. The lists of chemical radicals and chemical reactions were extended according to Nikjoo et al (2006). The chemical reactions in the pure water were considered as a surrogate of biological tissue. As directly introducing oxygen molecules in MC simulations increases the time costs dramatically, an approximation method was adopted in this work to simulate oxygen depletion reactions. The dissolved oxygen is regarded a homogeneous continuum, and the probability that a chemical radical reacts with the oxygenated continuum in time \( t \) can be calculated as

\[
P(t) = 1 - \exp \left( -4\pi D_{num} R_C C_o \left( t + 2R_C \sqrt{\frac{t}{\pi D_{num}}} \right) \right)
\]

where \( D_{num} \) is the sum of the diffusion coefficients of oxygen and the radical, is the reaction radius between oxygen and the radical, \( C_o \) is the concentration of dissolved oxygen (mol l\(^{-1} \)), \( M \), and it was calculated with \( C_o = pO_2 \times H_0 \) where \( H_0 = 1.73 \mu M \) mmHg\(^{-1} \) is the coefficient of Henry’s Law for oxygen dissolving in water (Colliaux et al 2015, Boscolo et al 2020). If the oxygen depletion reaction occurs, then the radical is replaced by the corresponding reaction product; for example, in a reaction between \( e_{aq} \) and \( O_2, e_{aq} \) is replaced by \( O_2^- \) (please see the supplementary material (available online at stacks.iop.org/PMB/66/185009/mmedia) section S1 and S2 for more details).

The impact of radical life time on the amount of oxygen depleted in FLASH irradiation (\( L_{SOD} \)) in the unit of \( \mu M \) Gy\(^{-1} \) was carefully studied, and the detailed analysis can be found in the supplementary material section S3. In this work, the lifetime values of H, \( e_{aq} \), and \( OH \) were set to 1 μs, 40 μs, and 1 ns, respectively, for simulations of FLASH irradiation.

### 2.2. Mathematical modeling of oxygen diffusion after FLASH irradiation

#### 2.2.1. Oxygen tension distribution in tissues at steady conditions

The mathematical model developed by Grimes et al (2014) was adopted in this work to describe the oxygen tension distribution in tissues at a steady state; that is, the state in which the tissues are not irradiated, or long
enough after FLASH irradiation, and the oxygen tension is already recovered. The blood vessel was simplified as having a cylindrical geometry with a radius of \( r_0 \), and oxygen diffusion from the blood vessels was considered to occur in a direction perpendicular to the vessel walls, being able to reach a maximum distance of \( r_r \); thus, the \( pO_2 \) distribution for \( r_0 \leq r \leq r_r \) at a steady state, \( p(r) \), can be described as:

\[
0 = D_{O_2} \left( \frac{1}{r} \frac{dp}{dr} \right) - C_{meta}
\]

wherein, \( D_{O_2} = 2.1 \times 10^{-9} \text{ m}^2 \text{ s}^{-1} \) is the diffusion rate of oxygen (Nikjoo et al 2006), and \( C_{meta} = 15 \text{ mmHg s}^{-1} \) is the consumption rate of oxygen due to metabolism (Grimes et al 2014), and equation (4) has two boundary conditions of \( p(r = r_0) = p_0 \) and \( p'(r_r) = 0 \); thus, the analytic solution of equation (4) is given by:

\[
p(r) = p_0 + \frac{C_{meta}}{4D_{O_2}} \left( r^2 - r_0^2 - 2r_r^2 \ln \frac{r_r}{r_0} \right)
\]

### 2.2.2. Oxygen tension recovery after FLASH irradiation

The oxygen tension in tissues can be changed due to ROD of FLASH irradiation and will gradually recover after irradiation due to oxygen diffusion from vessels, and the dynamic change of \( pO_2 \) in the recovery process can be described as:

\[
\frac{dp}{dt} = D_{O_2} \left( \frac{1}{r} \frac{dp}{dr} \right) - C_{meta}
\]

Equation (6) has two boundary conditions: the initial \( pO_2 \) distribution immediately after the FLASH pulse and the final \( pO_2 \) distribution after a full recovery; the initial \( pO_2 \) distribution is the \( pO_2 \) distribution described by equation (5) minus the amount of oxygen depleted during irradiation; and the final \( pO_2 \) distribution is the \( pO_2 \) distribution at steady state described by equation (5). MATLAB (Mathworks Inc.) was used to solve equation (6) using the finite differential method.

### 2.3. Modeling of cellular response after irradiation

In this work, two biological endpoints, the DNA damage yields and cell survival fractions (SFs) of the Chinese hamster ovary (CHO) cell line at different initial oxygen levels after CONV and FLASH irradiation were calculated and compared. The FLASH pulse was assumed to have an ultra-short pulse length and primary particles were assumed to incident instantaneously, therefore the intertrack chemical reactions were considered for FLASH pulse. The primary particles of CONV irradiation were considered to be separated, and they were simulated independently. DNA damage yield was calculated with the previously developed and well-validated DICOLDD (different cell oxygen level DNA damage) model (Zhu et al 2021); the DICOLDD model was developed based on OFH, and can be applied for DNA damage calculation under different cellular oxygen tension; cell SFs were calculated using a mechanistic cellular survival model developed by Wang et al (2018), which connected the relationship between radiation-induced double strand break (DSB) in the nucleus and the probability of cell survival. A brief introduction of the DICOLDD model and the mechanistic cellular survival model can be found in the supplementary material sections S4 and S5, respectively.

An index of radiosensitivity ratio (RSR) was defined to quantify the impact of ROD in FLASH. RSR was calculated as the ratio between doses under FLASH irradiation and under CONV irradiation needed to achieve the same biological effect (e.g. a given level of DNA damage or cell killing), and RSR describes the increment of dose tolerance due to oxygen depletion under FLASH irradiation

\[
RSR = \frac{D_{FLASH}}{D_{CONV}}
\]

### 2.4. Impact of initial oxygen tension and FLASH pulse delivery strategy on cellular responses

The impact of initial cell oxygen tension, FLASH pulse number, FLASH pulse interval, and radiation quality of source particles on the cellular response (DNA damage yields and cell SFs) was investigated and compared.

Our previous calculation (Zhu et al 2021) showed that the change in OC only had a limited impact on the DNA damage yield when OC was higher than 5%. However, when OC decreased below 1%, the DNA damage yield changed significantly with the OC. Experimental results (Berry and Stedeford 1972, Adrian et al 2020) and computational predictions (Pettersson et al 2020, Pratx and Kapp 2019a, 2019b) showed that the FLASH effect may only be observed in cells at a relatively low oxygen tension (<15 mmHg). Adrian et al measured SFs of prostate cancer cells at different oxygen levels (OC = 1.6%–20%) after CONV and FLASH irradiation. Their experimental results showed no difference between FLASH and CONV under normoxic conditions; however, a significant increase in SF after FLASH irradiation was found in the high dose region (18 Gy) in hypoxic cells.
### 3. Results

#### 3.1. Oxygen depletion during FLASH irradiation

$L\text{ROD}$ was simulated in cells with a different initial $pO_2$, and a typical FLASH pulse (4.5 MeV electron with a total dose of 10 Gy) was considered. Simulated $L\text{ROD}$ increased with the initial $pO_2$ of the tissue, with $L\text{ROD} = 0.38, 0.39, 0.41,$ and $0.43 \mu\text{M Gy}^{-1}$ (equivalent to 0.22, 0.23, 0.24, and 0.25 mmHg Gy$^{-1}$), when $pO_2 = 7.5, 15, 37.5$ and 160 mmHg, respectively.

$L\text{ROD}$ can be experimentally measured using solutions pre-equilibrated at a certain level of oxygen, and then sealed with glass to avoid gas exchanges during irradiation. Weiss et al measured oxygen depletion using oxygen-equilibrated bacterial cell suspension contained in sealed glass vessels and reported $L\text{ROD} = 0.58 \pm 0.1 \mu\text{M Gy}^{-1}$ under conventional dose rate irradiation of $^{60}\text{Co}$ and $L\text{ROD} = 0.26 \pm 0.05 \mu\text{M Gy}^{-1}$ at ultra-high dose rate exposure of 0.45 MeV electron in single pulses of about 3 ns (Weiss et al 1974). Michaels et al measured oxygen depletion using solutions irradiated with 25 MV x-rays and reported an $L\text{ROD} = 0.44 \mu\text{M Gy}^{-1}$ (Michaels 1986). The $L\text{ROD}$ values simulated here are in line with the experimental results.

#### 3.2. Oxygen distribution in biological tissues

Equation (5) was used to calculate the $pO_2$ distribution in the tissues around the vessels at a steady state, and the $pO_2$ within the vessel was set to $pO_2 = 100$ mmHg for the veins and $pO_2 = 40$ mmHg for the arteries. Following previously reported definitions (Liu et al 2015, Hall and Giaccia 2018), we delimited radiobiological hypoxia as $pO_2 < 3$ mmHg and pathological hypoxia as $3$ mmHg $< pO_2 < 15$ mmHg. Figure 1 shows that tissues that are $48 \mu\text{m}$ away from the center of the artery are exposed to pathological hypoxia, those that are $77 \mu\text{m}$ away from the center of the artery experience radiobiological hypoxia, those that are $20 \mu\text{m}$ away from the center of the vein experience pathological hypoxia, and those $45 \mu\text{m}$ away from the center of the vein experience radiobiological hypoxia.

Figure 1 (B) shows the dynamic change of $pO_2$ in the tissues around the vein ($pO_2 = 40$ mmHg within the vessel) after irradiation by a 4.5 MeV electron single FLASH pulse with a total dose of 10 Gy. As seen, tissues closer to the vein center show a more rapid decrease in $pO_2$ after irradiation due to a higher initial $pO_2$, and a...
faster recovery of pO2 due to a faster oxygen diffusion supplement from the vessels. The pO2 values dropped to the lowest level within 20 \( \mu \)s due to oxygen depletion. The low pO2 level was maintained for 1–10 ms, and the oxygen tension in tissues was generally fully recovered within 1 s.

### 3.3. Impact of initial oxygen level on the cellular response

In the following sections, the cellular responses to 4.5 MeV electrons delivered using CONV of FLASH mode were considered. The DNA damage yield induced by 4.5 MeV electrons was simulated using NASIC and the initial DSB radical yield after radiation was \( n_0 = 5.0 \text{ Gy}^{-1} \text{ Gbp}^{-1} \), and the initial direct DSB yield after irradiation was \( N_{dir} = 2.8 \text{ Gy}^{-1} \text{ Gbp}^{-1} \). Owing to the ROD in FLASH irradiation, the oxygen tension in cells decreased after FLASH irradiation, leading to a reduced indirect DSB yield (equations (S7)–(S10) in the supplementary material) compared with CONV irradiation.

A significant difference was observed in indirect damage yield induced by CONV and FLASH irradiation in cells with a lower initial oxygen tension (figure 2(A)). For cells with an initial oxygen tension of pO2 = 15 mmHg, the indirect DSB yields induced by 10 Gy FLASH irradiation and CONV irradiation were 3.40 and 3.57 Gy\(^{-1}\) Gbp\(^{-1}\), respectively, with a relative difference of 4.8% (=1–3.40/3.57); whereas for cells with an initial oxygen tension of pO2 = 7.5 mmHg, the indirect DSB yield induced by 10 Gy FLASH irradiation and CONV irradiation were 2.39 and 2.82 Gy\(^{-1}\) Gbp\(^{-1}\), respectively, with a relative difference of approximately 15.3% (=1–2.39/2.82). The indirect DSB yield per Gy per Giga basepairs (Gbp) of DNA decreased with reduced initial cellular pO2 in both FLASH and CONV irradiation, this is because hypoxic cells are more radioresistant and indirect DNA damages induced by hydroxyl radicals (\( \cdot \)OH) maybe repaired by thiols rather than ‘fixed’ by oxygen molecules (Zhu et al 2021). Besides, indirect DSB yield also decreased with increasing FLASH dose since FLASH irradiation continuously depletes cellular oxygen thus making cells more hypoxic with increasing dose and reducing indirect DNA damages.

Reduced DNA damage after FLASH irradiation can result in higher cell SFs, and figure 2(B) shows the calculated SFs (equations (S11)–(S12) in the supplementary material) of CHO cells at a different initial oxygen level that were exposed to 4.5 MeV electrons delivered using CONV (dashed lines) or FLASH (solid lines) mode. Although a different cell line was considered in our calculation, our results are in line with those of Adrian et al (2020). For CHO cells at a higher oxygen tension (>37.5 mmHg), no difference in SFs was observed between CONV and FLASH. In contrast, increased SFs are observed under FLASH irradiation in hypoxic cells, and an enlarged difference in SFs between CONV and FLASH irradiation was observed with increasing dose.

To quantify difference in cell SFs after CONV and FLASH irradiation, RSR values were calculated using equation (7) to describe the increased dose tolerance after FLASH irradiation to reach the endpoint of SF = 1%. RSR values were labeled in figure 2. For CHO cells at a relatively high oxygen tension (>37.5 mmHg), RSR were very close to 1, and no increase in dose tolerance after FLASH irradiation was observed. For CHO cells with an initial pO2 of 7.5 mmHg, RSR increased to 1.21, which means that a 21% higher dose is required under FLASH irradiation to reach the endpoint of SF = 1%, compared with CONV irradiation.

![Figure 2](image_url)

**Figure 2.** Indirect DSB yield (Gy\(^{-1}\) Gbp\(^{-1}\))(A) and survival fractions (B) of CHO cells with different initial pO2 values after being exposed to 4.5 MeV electron CONV irradiation (dashed line) or single FLASH pulse (solid line).
3.4. Impact of FLASH pulse characteristic (pulse number and pulse interval) on the cellular response

To investigate the impact of FLASH pulse fractionation on the cellular response, CHO cells (initial pO₂ = 7.5 mmHg) were exposed to 4.5 MeV electron FLASH irradiation with a total dose of 10 Gy delivered in 1, 2, 5, and 10 pulses. Figure 3(A) shows the dynamic changes in cellular pO₂ within 1 s; pO₂ values decrease rapidly during FLASH irradiation and gradually recover after irradiation. When cells were irradiated in the multi-pulse irradiation mode, the cell oxygen tension recovered partially during the pulse interval, and the lowest pO₂ level after a single FLASH pulse radiation could not be reached, which led to differential cellular responses. Table 1 summarizes the responses of CHO cells after irradiation; the average value of pO₂ after a single pulse was 21% lower than that after 5 pulses, and this further led to a 5.4% lower indirect DNA damage yield and a 16% higher SF.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Change of pO₂ in cells with an initial pO₂ of 7.5 mmHg exposed to 4.5 MeV electron FLASH irradiation. (A) A total dose of 10 Gy delivered in 1, 2, 5, or 10 pulses in 1 s. (B) 10 Gy delivered in 10 pulses which were separated by different intervals.

**Table 1.** The average pO₂, total DNA damage yield, and cell survival fractions of CHO cells (initial pO₂ = 7.5 mmHg) were exposed to 4.5 MeV electron FLASH irradiation with a total dose of 10 Gy delivered in 1, 2, 5, and 10 pulses; or 10 Gy delivered in 10 pulses which were separated by different intervals.

| Number of pulses delivering 10 Gy | Average pO₂ after irradiation (mmHg) | Total DSB yield (Gbp⁻¹ 10 Gy⁻¹) | SFs |
|---------------------------------|--------------------------------------|---------------------------------|-----|
| 1                               | 5.3                                  | 51.94                           | 0.140 |
| 2                               | 6.33                                 | 54.13                           | 0.122 |
| 5                               | 6.67                                 | 54.75                           | 0.118 |
| 10                              | 6.61                                 | 54.61                           | 0.119 |
| Maximum relative difference     | 21%                                  | 5.1%                            | 15.7% |

| Pulse interval (ms) | Average pO₂ after irradiation (mmHg) | Total DSB yield (Gbp⁻¹ 10 Gy⁻¹) | SFs |
|---------------------|--------------------------------------|---------------------------------|-----|
| 10                  | 6.31                                 | 54.02                           | 0.123 |
| 25                  | 6.35                                 | 54.10                           | 0.122 |
| 50                  | 6.44                                 | 54.28                           | 0.121 |
| 200                 | 6.85                                 | 55.06                           | 0.115 |
| 1000                | 7.28                                 | 55.82                           | 0.110 |
| Maximum relative difference | 13%                              | 3.2%                            | 10.6% |

*The maximum relative difference was calculated using: (1 − minimum/maximum) × 100%.
As shown in figure 1(B), the low pO2 level caused by ROD was maintained for milliseconds to tens of milliseconds, after which pO2 started to recover. Different pulse intervals result in different initial pO2 for the next pulse and changes the cellular responses accordingly. The impact of pulse interval on the cellular response was qualified by irradiating CHO cells (initial pO2 = 7.5 mmHg) with 10 × 1 Gy of 4.5 MeV electron FLASH pulses, and each pulse was separated by 10, 25, 50, 200, and 1000 ms; the dynamic change is shown in figure 3(B). Pulse interval of lower than 50 ms had a limited impact (<2%) on the cellular responses, but the cell SF decreased significantly when the pulse interval was extended to 1000 ms, due to the full recovery of oxygen tension during pulse intervals.

A similar analysis for the impact of pulse fractionation and pulse interval on the cellular response was performed for CHO cells with an initial pO2 of 15 mmHg, and the results have been provided in table S5.

3.5. Impact of radiation quality on the cellular response
Radiation quality can modify the cellular responses after FLASH irradiation in different ways; on one hand, high LET radiations can lead to higher DNA damage yields and induce more complex DNA damage distributions that are less likely to be repaired accurately and may result in chromosomal aberrations and lead to cell death. On the other hand, high LET radiation deposits more energy locally and produces chemical radicals with a denser spatial distribution, which makes it easier for radicals to react with each other and reduces the probability of reactions between dissolved oxygen molecules and eaq, as well as H; thus, a high LET radiation has lower oxygen depletion capability and the difference between the cellular responses induced by a high LET radiation in CONV and FLASH mode would also be reduced.

Figure 4 shows LROD values increase with pO2, but decrease with radiation quality. The LROD of 4.5 MeV electrons (LET = 0.3 keV/μm) reached 0.43 μM Gy⁻¹ (0.25 mmHg Gy⁻¹) at 760 mmHg, which was 38.9% higher than that of the 1.0 MeV protons (LET = 32.4 keV/μm⁻¹). Figure 4(B) shows low LET radiations show enlarged increments of SFs after FLASH irradiation, compared with a high LET radiation. The RSR value was 1.03 for 1.0 MeV protons, whereas it increased to 1.21 for 4.5 MeV electrons when an SF = 1% was adopted as the endpoint, indicating that a low LET radiation shows a better FLASH sparing effect on cell survival.

4. Discussion
In this study, we used MC simulations and analytical calculations to investigate the modified cellular responses in hypoxic normal cells under FLASH irradiation due to ROD, and our results show that at lower the initial oxygen tension, a more evident modified cellular response (higher RSR value, which means a more obvious radioprotective effect) can be observed after FLASH irradiation. One may deduce that hypoxic cancer cells are also prone to having a radioprotective effect after FLASH irradiation, and this deduction seems to contradict the published experimental results, which have shown that FLASH irradiation is as effective as CONV irradiation in killing tumor targets (Favaudon et al 2014, Diffenderfer et al 2020, Levy et al 2020).

It should be noted that, according to the simulated and experimental LROD, when human tissues (pO2 = 1–100 mmHg, corresponding to Cₐ = 1.73–173 μM) are exposed to a typical FLASH pulse (4.5 MeV
electron with a total dose of 10 Gy), less than 4.29 μM (2.5 mmHg) oxygen can be depleted during irradiation. Previous studies have shown that oxygen tension has a very limited impact on the cellular response when pO2 is higher than 30–40 mmHg (Hall and Giaccia 2018); however, the DNA damage yield (Zhu et al 2021) and cell SF (Ling et al 1981) changes significantly when pO2 is lower than 7.5 mmHg. This indicates that the change in cellular response due to oxygen depletion under FLASH irradiation can only be observed in cells at a relatively low oxygen level, such as cells located far away from the vessel center or hypoxic stem cell niches in normal tissues. Studies have shown that stem cell niches in the bone marrow experience local oxygen tension of 9.9–32 mmHg (Spencer et al 2014), that the pO2 of mesenchymal stem cells ranges from 15 to 60 mmHg, and the pO2 of hematopoietic stem cells ranges from 7 to 50 mmHg (Mohyeldin et al 2010). Pratx and Kapp proposed that hypoxic stem cells may be related to radioprotection from ROD associated with the FLASH effect (Pratx and Kapp 2019a, 2019b). Therefore, in this work, we focused on the cellular response of hypoxic cells.

The mechanism underlying the FLASH effect remains unclear and many different factors, such as the immunological signaling, modified microenvironment, and ROD effect, may act collectively to affect the FLASH effect (Zhou 2020). In this work, we only investigated the impact of ROD on the cellular responses after FLASH irradiation and other possible influencing factors were not considered. Therefore, the radioprotective effect resulting in ROD may be canceled out or enhanced by other factors in realistic biological tissues. For instance, the differential response to ROS between tumor cells and normal cells can result in different cellular responses. ROS, including O2-, H2O2, and OH-, are byproducts of aerobic metabolism. Low to moderate levels of endogenous ROS are required for cell proliferation, differentiation, and survival, and a mild increase in the ROS levels may result in a transient alteration in these activities; however, when the ROS level exceeds the threshold level, it may overwhelm the antioxidant capacity and result in cell death (Trachootham et al 2009). Cancer cells have higher levels of endogenous ROS compared to their normal counterparts due to the increased metabolism. When the redox balance in cells is disturbed, for example, when cells are exposed to FLASH irradiation, enormous amounts of ROS are produced due to radiochemical reactions (Labarbe et al 2020, Spitz et al 2019, Montay-Gruel et al 2019). It is easier for cancer cells to reach the ROS threshold that can selectively kill them but spare normal cells. Therefore, cancer cells are protected by ROD but are selectively damaged due to excessive ROS generation after FLASH irradiation. These two factors may cancel out the radioprotective effects of ROD.

5. Conclusions

In this work, the impact of ROD on the cellular response under FLASH irradiation was studied using MC simulations and mathematical modeling. The MCTS code NASIC was updated to support the simulation of oxygen depletion reactions. The calculated L_{ROD} values in a normal human tissue (pO2 = 1–100 mmHg) were lower than 0.43 μM Gy^{-1} (0.25 mmHg Gy^{-1}), which indicated that the modified cellular response due to oxygen depletion in FLASH irradiation can only be observed in hypoxic cells (pO2 < 30–40 mmHg).

We have systematically evaluated the impact of the initial oxygen tension, FLASH pulse number, pulse interval, and radiation quality of source particles on the cellular response. An index of RSR was proposed in this study to quantify the difference in cellular response after CONV and FLASH irradiation. Based on our calculations, we infer the following:

1. The FLASH radioprotective effect due to ROD is only evident in cells with a low pO2.
2. The multi-pulse delivery strategy is not preferred for obtaining a better radioprotective effect under FLASH irradiation because the cellular pO2 can be partially, or completely, recovered during pulse interval.
3. If the single pulse delivery regimen is not available in the practical application of FLASH irradiation, a pulse interval of less than 10–50 ms is suggested to take advantage of the oxygen tension preservation window and superpose the ROD caused by different pulses.
4. Low LET source particles, such as electrons, x-rays, and high-energy protons are preferred in FLASH irradiation to provide a better radioprotective effect to normal cells.

Our conclusions can serve as a reference for future FLASH applications, and the method developed in this work is applicable for prediction and comparison of future in vitro experimental results. Analyses that incorporate ROD, as well as other possible influencing factors, should be performed to better understand the FLASH effect. More experimental and theoretical analyses are needed to ascertain the biological mechanism of FLASH and promote the clinical application of FLASH radiotherapy.
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