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COMMENTARY

Radiotherapy Using High-Intensity Pulsed Radiation Beams (FLASH): A Radiation-Chemical Perspective

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Radiation chemists have been routinely using high-dose microsecond-pulsed irradiation for almost 60 years, involving many thousands of studies, in the technique of "pulse radiolysis". This involves dose rates broadly similar to the FLASH regimen now attracting interest in radiotherapy and radiobiology. Using the experience gained from radiation chemistry, two scenarios are examined here that may provide a mechanistic basis for any differential response in normal tissues versus tumors in FLASH radiotherapy. These are: 1. possible depletion of a chemical critical to the response to radiation, and 2. radical–radical reactions as a possible cause of effects occurring mainly with high-intensity pulsed radiation. The evidence for changes in relative levels of so-called "reactive oxygen species" produced after irradiation using FLASH versus conventional irradiation modalities is also examined. **© 2020 by Radiation Research Society**

INTRODUCTION

There has been considerable interest recently in the use of ultra-high-dose-rate radiotherapy, with reports of reduced normal tissue damage compared to conventional treatment (*1–14*). Typically, the newer modality, known as FLASH, might deliver a dose of a few Gy in one or a few pulses each of 1–2 μs duration separated by a fraction of a second, while the conventional delivery involves either steady radiation or a continuous train of low-intensity pulses, each typically delivering $<<0.1$ Gy. [Actual doses and doses rates vary considerably; the values indicated here were used in one typical laboratory study (*7*), described in more detail below.] There is the potential for misunderstanding when comparing the two regimens; thus, a recent editorial noted ''FLASH radiation . . . liberates significantly more electrons, resulting in many more ionization events than at conventional dose rates'' (*15*), reflecting discussion in one analysis (*10*). Of course, for the same overall *dose*, the number of ionizations is the same; what is different is that high-dose *rates* will generate a higher concentration of free radicals within a short time interval than the conventional treatment. This can alter the chemical pathways leading to cell death and tissue damage. (It is very important when comparing irradiation regimens using repetitive pulsed beams to give both the dose rates within each pulse and the inter-pulse separation, as well as the overall time-averaged dose rates.) The doses in microsecond pulses are not high enough for track overlap effects to be a factor, so apart from radical concentrations, the initial radiation chemistry is similar in both modalities, with similar *yields* of radicals ''escaping'' track recombination events in the first 10^{-7} s after ionization.

Radiation chemists have been routinely using a FLASH regimen of single pulses of a few Gy in approximately 1 μs or less in thousands of studies since 1960. The technique of ''pulse radiolysis'' has revolutionized knowledge since it allowed monitoring radiation-produced free radicals in real time. Thus, the general chemical consequences of high-dose-rate pulsed irradiation are very familiar to radiation chemists. Therefore, this perspective is offered as a guide to the types of mechanisms that might be involved and the possible effects of variables in FLASH other than dose rate, such as the inter-pulse interval. Some aspects have been discussed briefly by Koch (*16*), who also noted additional and important physiological factors.

POSSIBLE DEPLETION OF A CHEMICAL CRITICAL TO RADIATION RESPONSE

Two effects of high dose/dose rates are frequently encountered in radiation chemistry. One effect has long been appreciated in radiation biology. This is the depletion of a solute by radiation-chemical reactions when replenishment by diffusion is inefficient, commonly experienced

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when oxygen-containing systems are irradiated. This was observed in irradiated bacteria over 60 years ago (*17*) and is an effect that leads to ''break points'' in plots of cell survival versus dose, as cells initially oxygenated become anoxic; the use of two radiation pulses separated by a defined interval enabled observation of both oxygen depletion and its replenishment by diffusion (*18*). The possible depletion of tissue oxygen in the FLASH regimen has been discussed elsewhere (*3, 10, 11, 13, 19*). In principle, one could estimate doses that would significantly influence oxygen-dependent radiosensitivity via oxygen depletion, either by using the survival break points observed for mammalian cells *in vitro* or by calculating the consumption of oxygen by radiation-produced free radicals; the yields of the latter are well-established in pure water with well-defined solutes (*20*).

A calculation of radiolytic oxygen depletion was attempted recently (*10*), but it was assumed oxygen scavenges hydrated electrons (e_{aq}^-) and $H^\bullet$ atoms as a significant route to deplete O_2 in cells. Another calculation in this context stated that ''oxygen can be depleted during irradiation due to its reactions with ... the solvated electron and the hydrogen radical'' (*19*), and a further modeling study (*21*) correlated yields of superoxide from these primary water radicals in pure water with the oxygen enhancement ratio in radiobiology. All three studies overlooked the fact that reactions of e_{aq}^- or $H^\bullet$ with O_2, while important in the radiolysis of pure, oxygenated water, probably occur to a very small extent in cells because of the high concentrations of competing scavengers. To illustrate this, we can calculate the ''scavenging capacity'' (rate constant $k \times$ concentration) for the reaction of e_{aq}^- with 20 μM O_2 (partial pressure $pO_2 \approx 1.6$ kPa/12 mm Hg) in water at ambient temperatures, $k[O_2] \approx 3.8 \times 10^5$ s^{-1} (*22*). This may be compared with the scavenging capacity for e_{aq}^- of major cellular constituents, estimated to be approximately 1,000-fold higher at $\approx 3.4 \times 10^8$ s^{-1} (*23*). While this simple comparison ignores cellular heterogeneity, it is at least more realistic than modeling the cell as pure water.

In irradiated cells or tissue, oxygen is therefore likely to be consumed largely via the formation of transient peroxyl radicals ($ROO^\bullet$) formed in diverse secondary reactions; some peroxyl radicals can dissociate to superoxide radicals (*24, 25*) and thus partially re-supply O_2 as well as produce hydrogen peroxide (see below). In practice, the timescale and extent of oxygen consumption is impossible to estimate reliably for the complex mixture of radicals that might be formed in cells, both because the timescales and efficiencies of superoxide elimination vary widely (*26*), but especially since both thiols and ascorbate are likely to modify the pathways involved (as does, of course, superoxide dismutase, catalase and other peroxidases). The interaction of thiols, oxygen and ascorbate in radical chemistry has been outlined elsewhere (*27*). Briefly, diverse radical ''repair'' reactions of thiols (including reaction with peroxyl radicals) necessarily generate another radical (thiyl), which in turn conjugate with thiols in an equilibrium, producing disulfide radical-anions (*28*), the latter reacting rapidly with O_2 yielding superoxide; however, ascorbate scavenges thiyl radicals efficiently to intercept this pathway (*29*). These complications also suggest that because cells cultured *in vitro* generally lack ascorbate, the break points discussed above are unlikely to be translated quantitatively to tissues *in vivo* even if the cells were otherwise thought to be good models. While oxygen depletion is easily measured in irradiated cell culture media (*30*) or stirred cell suspensions (*31*), because of the diverse chemical pathways outlined above, these measurements cannot be translated to living tissue.

However, the analysis by Spitz *et al.* (*10*) proposed that most of the oxygen consumed in irradiated tissue would result from lipid peroxidation rather than $e_{aq}^-/H^\bullet$, chain reactions amplifying oxygen consumption. This raises the important question of the *timescale* of oxygen depletion. As was emphasized elsewhere (*32*), time is an important variable in the ''oxygen effect'' in radiobiology. While over 50 years have passed since the ground-breaking work defining the timescale of the oxygen effect (*33–35*), these time-resolved studies are central to any discussion of oxygen depletion and FLASH regimens. Lipid peroxidation is a relatively slow reaction: While the timescale in human tissue is uncertain, the product of rate constant and target lipid concentration suggested in one model implies a half-life for the critical chain propagation step of approximately 0.4 s (*36*), a half-life which may be an underestimate if the rate constant of this step is only 10 M^{-1} s^{-1} (*37*). This suggests that significant oxygen depletion via lipid peroxidation probably occurs on a timescale much longer than the critical ''window'' of 1–2 ms (*35*) that is available for modification of oxygen-sensitive radiation damage associated with cell survival. In short, lipid peroxidation *may* be of secondary importance if oxygen is depleted after the oxygen-sensitive radicals on DNA (or other target) have decayed to paths that cannot be intercepted by oxygen: It is no use shutting the door after the horse has bolted. Thus, even if lipids do act as a ''sponge'' for oxygen and show consumption of oxygen enhanced by chain reactions, it is vital to compare the timescale of oxygen consumption in radiation-induced lipid peroxidation with the timescale of the ''oxygen effect'', as well as consider diffusion times of oxygen even within the cell.

Incidentally, model systems of radiolysis of lipid micelles or liposomes in aqueous suspension have reported an *inverse* dose-rate effect on peroxidation or oxygen consumption (*38–40*), as was previously mentioned by Koch (*16*). This needs to be carefully considered if lipid peroxidation is postulated to be a significant source of oxygen depletion in FLASH regimens. One might expect peroxidation to be *less* at high-dose rates since the greater importance of radical–radical reactions implies an enhancement of chain-terminating reactions. Nonetheless, lipid

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peroxidation can be deleterious (*41*) and the topic merits further measurements in radiobiology.

However, we cannot yet dismiss lipid peroxidation as a modulating factor in FLASH because the timescale of oxygen depletion by it appears to be much too slow. If the regimen involves two or more pulses, then slow oxygen-depletion pathways (of any type) may indeed become relevant *if the inter-pulse interval is longer than the time required for oxygen depletion*. The Einstein-Smoluchowski equation indicates the one-dimensional root-mean-square diffusion distance of a small molecule, with a diffusion coefficient in the cytoplasm of 2×10^{-9} m^2 s^{-1}, is 2 μm in 1 ms; while some time is required for oxygen consumption in lipids to be reflected throughout the cell, the lipid chain propagation step is still rate-determining. In the earliest published study (*42*) of a break point effect in pulse-irradiated mammalian cells, it was found that if a dose showing the change to anoxic behavior after a single radiation pulse was split into two pulses 2.5 ms apart, the break point was not seen, so this time interval was sufficient for O_2 to be replenished from the media. [However, it is important to take into consideration the discussion of this work by Berry *et al*. (*43*), especially the low doses at which the break point was observed.] Later double-pulse experiments greatly extended these observations (*18*), and the timescale of O_2 depletion was considered in detail (*44*). Of course, in tissues, both diffusion and perfusion properties govern O_2 supply, and translating the effects seen in dilute cell suspensions is more complex.

Importantly, when considering radiation-chemical depletion of oxygen in irradiated tissue, *the spatial and temporal development of oxygen loss must be considered*. This places considerable constraints on methods to measure oxygen levels in tissues after FLASH irradiation: using, e.g., an electrode, optode or luminescent ''reporter'' that has a time resolution longer than 1 ms (perhaps even less) may result in misleading conclusions, although any measurements in tissues may be better than nothing and will probably be better than predictions from radiation chemistry. Optical methods can ''report'' oxygen levels in the immediate vicinity of DNA: a particularly simple exploitation of standard commercial fluorimeter instrumentation to probe radiosensitizer, O_2 or thiol concentrations near DNA in cells *in vitro* has been described elsewhere by this author (*45, 46*). Although this simple methodology is unsuitable to apply to the FLASH problem *in vivo*, there is intense research activity in optical probes for O_2 levels. A fairly recent published review (*47*), partially updated in 2015 (*48*) and 2020 (*49*), cited 694 publications (385 even in the extensive supplementary material). Targetable nanosensors capable of resolving intracellular O_2 gradients are available and demonstrated differences between tumors and normal cells (*50*). Surely it is possible to make appropriate measurements of oxygen levels in tissue to address these issues in the context of FLASH.

Other candidate chemicals for rapid depletion by a high concentration of radiation-produced radicals may be considered. Break points in survival curves of irradiated bacteria in the presence of nitric oxide ($NO^\bullet$) were reported 50 years ago (*51*), although the studies merit revisiting given the experimental constraints at that time. The steady-state levels of $NO^\bullet$ in tissue are not known with confidence and may be a few nanomolar or less except in stress conditions such as inflammation; the levels needed to perform its function as ''endothelial derived relaxing factor'' are of the order of 10 n*M* (*52, 53*). While levels of $NO^\bullet$ in tissue will be *very* much lower than those of oxygen, like oxygen, $NO^\bullet$ is promiscuous in its high reactivity towards free radicals, possibly even more so, and thus it may be even easier to deplete by reaction with diverse radiation-produced radicals. Furthermore, apart from its effects on vascular tone and therefore on oxygen delivery, $NO^\bullet$ appears to be significantly more efficient as a hypoxic cell radiosensitizer than oxygen, at clinically-relevant radiation doses (*54*), acting via a mechanism different from oxygen (*55, 56*). [Some earlier work using $NO^\bullet$ may have been compromised by the artefact of direct cellular thiol depletion at moderate-to-high concentrations of $NO^\bullet$ (*57*).]

At first sight, $NO^\bullet$ would have to compete with O_2 for radiation-produced secondary radicals (except in anoxic cells), and its reactivity towards such radicals is unlikely to be higher than that of O_2 by a sufficient factor to overcome the concentration differences. However, $NO^\bullet$ reacts with almost diffusion-controlled rate constants with superoxide (*58, 59*), thiyl radicals ($RS^\bullet$) (*60*), lipid-derived (*61*) and other organic peroxyl radicals (*62*) and protein radicals (*63, 64*), so there are many pathways that could deplete $NO^\bullet$ even in the presence of oxygen. Radiation-induced hypoxia can itself lead to a rapid decrease of $NO^\bullet$ levels in macrophages, so $NO^\bullet$ levels can be modulated by FLASH less directly (*65*). If nitric oxide is a factor in radiobiology, there may again be differences between cultured cells and tissue, because of possible ascorbate involvement in the competing reaction pathways, such as reaction of DNA-derived radicals with ascorbate (*66*) rather than with $NO^\bullet$ (*54*). A basis for a therapeutic differential between normal tissues and tumors linked to $NO^\bullet$ is not immediately evident [although inducible nitric oxide synthase is overexpressed in some tumors compared to normal tissues (*67*)]. However, this largely speculative discussion serves both to remind us that there could be candidates other than oxygen for radiation-chemical depletion in FLASH regimens, and to highlight the need for better understanding of the role(s), if any, of $NO^\bullet$ in the radiotherapeutic response.

RADICAL–RADICAL REACTIONS AS A POSSIBLE CAUSE OF EFFECTS OCCURRING MAINLY WITH HIGH-INTENSITY PULSED RADIATION

Another effect of high-dose rates that radiation chemists using pulse radiolysis frequently encounter is a situation in

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which the effects of radical–radical reactions become important; these effects may be negligible when low-intensity or continuous-beam radiation is employed. In pulse radiolysis experiments competing reactions for a radical are a common occurrence, with one reaction type being with one or more solutes at considerably higher concentrations than the radicals, and the other involving the radical reacting with either the same or a different radical. The rates of the reaction of radicals with solutes are proportional to the radical concentration, but the rates of radical–radical reactions are proportional to the *square* of the radical concentration. Thus, while some radical–radical reactions may play a role in the FLASH high-dose-rate modality, they may not be important with conventional treatment. Koch has previously stated that ''... one cannot necessarily rule out additional radical–radical interactions at FLASH compared with conventional dose-rates.'' (*16*); and over 50 years ago high-dose-rate effects prompted Berry *et al*. to suggest: ''A high local radical concentration, which resulted in radical–radical interactions, could result in a reduced number of radicals remaining free to interact with the biological target in the presence of oxygen...'' (*68*).

Superoxide radicals, for example, may be involved in radical–radical reactions that modify radical damage to a critical target such as DNA, but that might only become a significant factor under high dose-rate irradiation. Two simple models suffice to illustrate this possibility, although it appears unlikely that the explanation for the FLASH effect is related to these specific examples, as noted below. Thus $^{\bullet}$OH radicals add to DNA bases to generate radicals $(DNA)^{\bullet}$ reactive towards oxygen; the resulting peroxyl radical may abstract H from a nearby sugar to eventually lead to a strand break, as has also been reported by Bamatraf and O'Neill (*69*) in the context of nitroaromatic ''oxygen-mimetic'' radiosensitizers:

$$(DNA)^{\bullet} + O_2 \rightarrow (DNA)OO^{\bullet} \quad (1)$$

$$(DNA)OO^{\bullet} \rightarrow (DNA)OOH + \text{sugar damage} \rightarrow \rightarrow \text{strand break.} \quad (2)$$

[Addition of a peroxyl radical to an adjacent base is also possible (*70*).] As discussed above, superoxide generation by breakdown of diverse peroxyl radicals with appropriate functionality, or produced via thiyl radicals and the resulting disulfide radical-anions reducing O_2, may occur in parallel and thus be available for partially preventing reaction [Eq. (2)] in a *radical–radical* reaction [Eq. (3)], perhaps only significant after sufficiently high intensity pulsed doses as in FLASH:

$$(DNA)OO^{\bullet} + O_2^{\bullet -} + H^+ \rightarrow (DNA)OOH + O_2. \quad (3)$$

Although some normal tissues exhibit mild hypoxia, this oxygenation is at a level higher than in the critical hypoxic fraction of cells in tumors [see, e.g. (*71, 72*)]. Therefore, somewhat higher levels of radiation-generated superoxide may be a feature in normal tissues compared to tumors, FLASH thus exhibiting some selectivity in damage sparing, an effect seen only when the dose is delivered in large pulse(s).

A critical test of a model for oxygen-dependent radiosensitization is competition with thiols influencing the kinetics and extent of damage, as demonstrated directly by Michael *et al*. for the thiol-dependent kinetics of the oxygen effect (*73–75*) and emphasized, in particular, by Koch *et al*. (*76, 77*). With reactions such as that of Eq. (1) or competing ''repair'' by thiols reacting with $(DNA)^{\bullet}$ (*78*) or $(DNA)OO^{\bullet}$ (*79*), the radical concentration is much less than either the O_2 or thiol concentrations, so the critical competition between oxygen and thiols is less likely to be affected by FLASH conditions than are radical–radical reactions.

One can easily envisage alternative models involving a radical–radical reaction that alters damage. Thus, the deprotonated radical from the positive ''hole'' [the guanine radical, $Gua(-H)^{\bullet}$] reacts with superoxide:

$$Gua(-H)^{\bullet} + O_2^{\bullet -} \rightarrow \text{hydroperoxide radical} - \text{adducts} \rightarrow \rightarrow \text{complex products,} \quad (4)$$

with a rate constant close to the diffusion-controlled limit in nucleosides and DNA. The eventual products of this reaction have been detected *in vitro*, and strand break formation in DNA originating from H abstraction at 2′-deoxyribose by $Gua(-H)^{\bullet}$ has been identified (*80–83*). [Note that superoxide is not simply acting as a ''repair'' agent (*84, 85*).] The competing reactions with thiols and ascorbate ($AscH^-$), reactions that will be much less dose-rate dependent, have been characterized (*66, 86, 87*):

$$Gua(-H)^{\bullet} + RSH(\text{or} + AscH^-) \rightarrow RS^{\bullet}(\text{or } Asc^{\bullet -} + H^+) + \text{products.} \quad (5)$$

Both of these examples involve superoxide as the protective reductant. In the cell, superoxide dismutase is available to counter such a role. However, its effectiveness might be diminished under conditions of high-intensity pulsed irradiation if superoxide originates near the target via dissociation of peroxyl radicals (for example), with the enzyme having limited accessibility. Again, spatial and temporal considerations are a factor, the heterogeneity of the cell greatly complicating the application of simple homogeneous kinetics to assess possible reaction pathways. The ''footprint'' of the products of Eq. (4) in the second example provides a route to exploring such possible mechanisms *in vivo* (*84, 88*), and this might also be possible after more detailed consideration of other radical–radical reactions.

It should be noted that reactions shown in Eqs. (1) to (3) are grossly oversimplified representations (*24, 25, 70, 82, 85, 89–92*). In any case, a contraindication to invoking radical–radical reactions involving superoxide in FLASH mechanisms is that one might then expect high dose rates to

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influence the magnitude of the oxygen enhancement ratio, contrary to observations, e.g., (*35*). Berry *et al.* concluded in 1973, ''. . .exposures to single pulses of *x* rays and high-energy electrons at dose-rates greater than [*ca.* 2×10^9 Gy s^{-1}] produce survival curves for mammalian cells which are very little different from those obtained after irradiation at conventional dose-rates around [0.02 Gy s^{-1}]'' (*43*), a conclusion supported by subsequent published work (*93*). Another study, utilizing pulsed electron beams for both high and low dose rates (4–6 μs pulses at 5-ms intervals, dose/pulse ≈ 1.6 Gy and 0.5 mGy, respectively), showed similar responses of hamster cells in both air and anoxia for the two regimens (*94*). However, most studies compare air-equilibrated cells with anoxic conditions, whereas cells at intermediate O_2 levels are most relevant. In contrast to mammalian cells, the radiation response of anoxic *Bacillus megaterium* spores in aqueous suspension increases at high dose rates (*95*), and differences between organisms with very different cellular structures may suggest other factors of possible importance. There are certainly dramatic dose-rate effects in mammalian cells at lower dose rates (*96*), but these reflect slow, biochemical repair pathways (*97*).

Thus, these two putative reactions, Eqs. (3) and (4), are offered merely as an illustrative starting point to stimulate further exploration rather than serving as plausible proposals. Further analysis of the role of superoxide dismutases in controlling superoxide levels at high dose rates is desirable, as is consideration of radical–radical reactions involving two peroxyl radicals (*26, 98, 99*). This commentary serves to highlight the parameters in which any radiation-chemical explanation of FLASH must work, with the use of a mechanism that encompasses a large amount of biological data, and which is chemically plausible. In addition, cellular heterogeneity and accompanying non-homogeneous kinetics must be addressed to work towards a full understanding.

THE INVOLVEMENT OF REACTIVE OXYGEN SPECIES (ROS) IN FLASH REGIMENS

A recently published study (*7*), entitled *Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species*, asserted ''. . .radiochemical studies confirmed that FLASH produced lower levels of the toxic reactive oxygen species hydrogen peroxide.'' (The vague term, ''reactive oxygen species'', and its acronym ROS are not often encountered in radiation chemistry, as it usually adds obfuscation rather than clarification.) Obvious concerns, such as the toxicity of hydrogen peroxide at levels produced by radiation and the cellular defenses against these ROS, are discussed below; first we examine the evidence presented in this study to support the central tenet reflected in the title.

The authors measured H_2O_2 yields in water containing 50 μ*M* O_2, reporting lower yields after irradiation by a few single, large pulses to the same dose compared to a train of many small pulses, with during-pulse dose rates of the order of 3×10^6 or 3×10^4 Gy s^{-1}, respectively. H_2O_2 was measured after radiolysis by 6-MeV electron pulses of unbuffered water (stored for 24 h in polypropylene tubes before irradiation) initially containing 50 μ*M* O_2. The FLASH modality used 5 Gy, 1.8 μs pulses, separated by 10 ms, repeated as necessary to obtain doses of 10–80 Gy. For the ''conventional'' procedure, used for comparison, 10 Gy irradiation was delivered in 350 pulses, each 1 μs, at 100 ms intervals (i.e., each pulse was 10/350 ≈ 0.03 Gy), and the cycle was repeated to obtain 10–80 Gy; the *average* dose rate overall was ≈0.3 Gy s^{-1}, but the during-pulse dose rate was still much higher than continuous irradiation with the same average dose rate. The measured yield of H_2O_2 was ≈ 0.15 μ*M* Gy^{-1} for conventional irradiation, reduced to ≈0.12 μ*M* Gy^{-1} for FLASH conditions.

Production of H_2O_2 from the radiolysis of water containing O_2 has been measured at least since 1928 and more extensively in the 1950s and 1960s with a wide range of pH, O_2 concentration, doses, dose rates, scavengers and types of radiation. Two independent studies in the 1960s, of pulse-irradiated oxygen-saturated water using dose rates between 5×10^6 and 5×10^7 Gy s^{-1}, reported H_2O_2 yields of ≈0.20 μ*M* Gy^{-1} (*100, 101*). The yield is considerably lower when low-dose-rate irradiation is used. Thus a recently reported study, which also included measurements after irradiation with protons and heavier ions, summarized literature data covering the entire pH range, and included new measurements for gamma radiolysis (*102*): the yield of H_2O_2 in air-saturated water around neutral pH and dose rate of ≈1 Gy s^{-1} was ≈0.10 μ*M* Gy^{-1}. Removal of O_2 reduces the yield of H_2O_2; low concentrations of added radical scavengers further reduce it to the ''escape yield'' of ≈ 0.07 μ*M* Gy^{-1}, and high concentrations of radical scavengers (but not those that may generate superoxide in secondary reactions) result in very little H_2O_2 production (*103*). Early work using gamma radiolysis showed that the water must be exhaustively purified, with meticulous cleaning of glassware, to avoid erroneous measurements (*104*).

The yields of H_2O_2 reported in the FLASH versus conventional comparison (*7*) therefore differ from what would be expected, unless repetitive pulsing introduces unanticipated effects. The use of plastic irradiation vessels, especially when samples are left in these vessels for 24 h before irradiation, is unprecedented in radiation chemistry; the artefacts arising from impurities discussed in several published studies between 1942 and the 1960s suggest the leaching of chemicals into the water as a possible complication in the recent measurements. Polypropylene and other plastic vessels can release chemicals into water even after a relatively short exposure (*105–107*).

To explore the effects of repetitive pulsing, the current author has performed calculations of the H_2O_2 yields in irradiated unbuffered neutral water containing the same O_2 concentrations and involving the precise radiation regimens, including pulse interval, as used in all the published studies

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described above. The calculations involved the integration of the differential equations characterizing the reactions occurring in irradiated oxygenated water, reactions which are all well-established, particularly the five dominating the production of H_2O_2 additional to the ''basal'' yield produced by in-track recombination of $^{\bullet}OH$, ≈0.07 μ*M* Gy^{-1}:

$$e_{aq}^{-} + O_2 \rightarrow O_2^{\bullet -} \quad (6)$$

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet} \quad (7)$$

$$O_2^{\bullet -} + HO_2^{\bullet}(+ H^+) \rightarrow O_2 + H_2O_2 \quad (8)$$

$$2^{\bullet}OH \rightarrow H_2O_2 \quad (9)$$

$$O_2^{\bullet -} + {}^{\bullet}OH + H^+ \rightarrow O_2 + H_2O. \quad (10)$$

The rate constants for reaction Eqs. (9) and (10) are of the same order, but that for Eq. (8) is over four orders of magnitude slower at pH ≈ 7 (*22, 108*). There are additional radical–radical interactions that may play a significant role only at low O_2 concentrations and high pulse doses. FACSIMILE code, based on the Gear algorithm, which has been used extensively in radiation chemistry, was employed to model overall 24 equilibria and reactions, in addition to the initial radical-generating steps, very similar to those performed by LaVerne *et al*. but excluding reactions relevant only at high pH arising from prototropic dissociation of $H^{\bullet}$, H_2O_2 and $^{\bullet}OH$ (*102, 109*).

The calculations provided estimates of the H_2O_2 yields within approximately 5% of those measured for both high- (*100, 101*) and low- (*102*) intensity irradiation, but suggested that the FLASH and conventional regimens in the recently reported study (*7*) would have yielded approximately 0.19 and 0.11 μ*M* Gy^{-1} H_2O_2, respectively, after 10 Gy irradiation. These values differ significantly from the measurements reported (*7*) and the calculations are not consistent with the claim of FLASH producing lower levels of H_2O_2. The FLASH regimen simulations were slightly sensitive to overall dose and pulse interval, because some $HO_2^{\bullet}/O_2^{\bullet -}$ persists for much longer times after the pulse than e_{aq}^{-}, $H^{\bullet}$ and $^{\bullet}OH$ in oxygenated water. Thus, immediately before the second and subsequent pulses there is some superoxide left to enhance the effectiveness of the reaction Eq. (10), slightly reducing the H_2O_2 yield.

Notwithstanding these differences (the exceptional sensitivity to impurities is a potential problem with *all* these measurements, and the effects of uncertainties in rate constants require analysis), the use of pure water as a model for studying H_2O_2 generation in irradiated tissue is obviously completely non-biomimetic. This is because the reactions of Eqs. (6), (7), (9) and (10) are unlikely to occur to a significant extent in irradiated cells because of the high radical scavenging capacities previously noted elsewhere (*23*). H_2O_2 is sourced mainly via reaction Eq. (8) (catalyzed by superoxide dismutases) after secondary radical reactions that produce superoxide, plus a ''basal'' level of ≈0.07 μ*M* Gy^{-1} H_2O_2 produced in the radiation track by reaction Eq. (9). The latter yield is much reduced in the presence of high concentrations of $^{\bullet}OH$ scavengers (*103*) and it will contribute equally in both modalities; the vast majority of the numerous radical-chemical studies of H_2O_2 yields focus on the concentration-dependent effects of added scavengers.

Even if FLASH produced less H_2O_2 than conventional irradiation, as has been suggested elsewhere (*7*), the initial levels shortly after irradiations at a few Gy are most unlikely to be more than a few micromolar, and will rapidly decrease because of peroxidase activity. Hydrogen peroxide produces quite different damage compared to ionizing radiation, and several studies of the effects of H_2O_2 without irradiation but in a radiobiological context indicate that significant cell kill or generation of DNA double-strand breaks requires exceptionally higher extracellular levels of H_2O_2 in typical *in vitro* studies than are likely to be produced by FLASH radiolysis in tissues (*110–112*). This suggests that the role of radiation-produced H_2O_2 in radiobiology is minimal and does not require serious consideration in the context of FLASH. In the last decade or so, intense activity has resulted in major advances toward understanding the roles of H_2O_2 in biology, with the application of increasingly sophisticated methodologies [e.g., (*113–117*)]. Extracellular:intracellular concentration gradients of H_2O_2 of ≈390 have been reported (*118*), reflecting rapid catalytic destruction. It would not be prudent to extrapolate the lack of cytotoxic effects in cell suspensions containing a few micromolar H_2O_2 in the media (or even considerably higher concentrations), after exposure of minutes or longer, to possible effects of low levels of H_2O_2 produced intracellularly in a microsecond pulse of radiation. One review, discussing the highly selective oxidation of peroxiredoxin thiol groups by H_2O_2, noted ''Basal cytosolic steady-state H_2O_2 concentrations are estimated to lie in the low nanomolar range (≈1–10 n*M*) … and to rise transiently to the upper nanomolar range during oxidative signaling events (≈500–700 n*M*)'' (*119*).

It does appear likely that radiolytic H_2O_2 production is at an exceedingly low level and rate to challenge cellular defenses, even if some is produced at a higher rate (or even extent) after FLASH irradiation. However, it is probably still worthwhile for radiation biologists to exploit the methodology now being applied in the field of redox biology, or at least the quantitative concepts now emerging, to map experimentally or (much easier) to compute the temporal distribution of *transient* levels of H_2O_2 in irradiated tissue, and assess the possible consequences in the context of recent advances.

It is logical to separate the H_2O_2 production into two quite distinct phases. The first phase is the bolus production of a basal level of H_2O_2 produced by track events; this production precisely mirrors the duration of the pulse(s) to within *ca*. 10^{-7} s and has a yield of approximately 0.03 μ*M* Gy^{-1} if the $^{\bullet}OH$ radical scavenging capacity is *ca*. 8×10^8 s^{-1}

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(*23*) [by comparison with other $^{\bullet}OH$ scavengers in aerated water (*102*)], with similar yields, regardless of dose rate. Effort has already been made to compare steady-state levels of H_2O_2 with the effects of bolus addition (*120, 121*), and a computational framework for modeling bolus addition of H_2O_2 is established (*122*). It should therefore be possible to assess the effects of generating micromolar H_2O_2 fairly uniformly within the cell in approximately 1 μs (*122, 123*). The second phase is the very much slower production of further H_2O_2 via secondary radical reactions mainly involving addition of O_2 and elimination of superoxide, in competition with complex reactions involving thiols and ascorbate. It appears likely that the cell can cope with this insult because of the high intracellular peroxidase activity; however, it is likely that this needs to be further checked by computation.

Incidentally, radiation chemistry has already contributed to the redox biology of superoxide/H_2O_2 in many ways, exploiting the ability to generate superoxide selectively and monitor radicals directly in real time, including exploring the possible saturation of superoxide dismutases by high levels of superoxide (*124*) and the effects of pH and salts on SOD activity (*125*). It seems timely to consider the role of these enzymes in responding to high radical concentrations in the context of FLASH. Pulse radiolysis has also been applied to study peroxidase mechanisms [e.g., (*126–130*)]. Peroxiredoxins are currently attracting much attention in the redox biology of H_2O_2 (*113–117*), and there have already been studies of these enzymes involving radiolysis methods (*131, 132*). The ability to generate H_2O_2 in a few μs with very-high-intensity pulses might help to further characterize the reactions of peroxiredoxins.

Further support for the involvement of lower levels of ROS after FLASH compared to conventional irradiation was based mainly on experiments involving the addition of 4–5 m*M* amifostine or *N*-acetylcysteine to zebrafish embryos (*7*). Assuming amifostine was dephosphorylated under the conditions used (*133*), adding high concentrations of thiols to cells *in vitro* produces a marked perturbation of the intracellular thiol status before irradiation takes place, via thiol/disulfide exchange (in addition to the exogenous thiol loading), and the polyaminothiol from amifostine can accumulate in cells to a substantial degree (intracellular concentrations much higher than those in the medium) because of pH-driven effects (*46, 76, 134*), artefacts not translatable *in vivo* because of the ''simple arithmetic'' of cell density differences (*135*). There can be effects of thiols on the oxygen concentration in the media without irradiation (*76*). Furthermore, thiols are not selective ROS scavengers: while they do indeed react extremely rapidly with $^{\bullet}OH$ radicals (and $H^{\bullet}/e_{aq}^{-}$) (*22*), much more slowly with $HO_2^{\bullet}/O_2^{\bullet-}$ (*108*), and generally *very* much more slowly with H_2O_2 (*115, 136*), they also react rapidly with diverse secondary radicals in well-known H-donation mechanisms (*24, 28, 76*). Thiols are also highly reactive towards radical centers that could be formed directly by radiation without the involvement of ROS at all, such as those associated with the positive ''hole'' $Gua^{\bullet+}$ [see reaction Eq. (5)].

Because of these complications, in the absence of further, more bio-mimetic measurements or appropriate calculations, there is currently insufficient evidence to support the assertion (*7*) that the neurocognitive benefits of FLASH irradiation are driven by lower levels of ROS production than in conventional modalities.

CONCLUSIONS

The irradiation conditions used in FLASH radiotherapy are similar to those used since 1960 in thousands of studies by radiation chemists for exploiting the pulse radiolysis technique. Such studies routinely raise issues over solute depletion at low concentrations, as well as controlling contributions of competing radical–radical reactions. This experience is relevant to determining explanations for differential effects of FLASH compared to low-dose-rate modalities. In both scenarios the timescale and spatial distribution of events must be considered, as well as variables such as the inter-pulse interval assessed in this context when multiple pulses are used. Radiation chemistry can also contribute to assessing the possible involvement of *specific* reactive oxygen species in such effects; indeed, the reactivities of all relevant ROS have been rather well characterized, mainly by radiation chemists, and there is neither need for, nor merit in using the term ROS in radiobiology at all.

An earlier article was entitled (tongue-in-cheek) *Radiation chemistry comes before radiation biology* (*137*), to which could be added: ''Get the first wrong, then you get the second wrong''. The current commentary does not provide answers to any of the mechanistic questions raised by the FLASH modality; but it is hoped that this may guide researchers towards fruitful investigations while discouraging them from pursuing avenues that lack a sound quantitative basis.

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