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Author(s)	Shiraishi, Yuta; Matsuya, Yusuke; Fukunaga, Hisanori
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3

4 **Authors:** Yuta Shiraishi^{1,2}, Yusuke Matsuya³, and Hisanori Fukunaga^{3*}

5

6 **Affiliations:**

7 ¹ Graduate school of Health Sciences, Hokkaido University, N12 W5 Kita-

8 ku, Sapporo, Hokkaido, 060-0812, Japan

9 ² Faculty of Health Sciences, Japan Healthcare University, 3-11-1-50

10 Tsukisamu-higashi, Toyohira-ku, Sapporo, Hokkaido, 062-0053, Japan

11 ³ Faculty of Health Sciences, Hokkaido University, N12 W5 Kita-ku,

12 Sapporo, Hokkaido, 060-0812, Japan

13

14 *Corresponding author: Hisanori Fukunaga

15 E-mail: hisanori.fukunaga@hs.hokudai.ac.jp

16

17 **Abstract**

18 FLASH radiotherapy (FLASH-RT) has great potential to improve patient
19 outcomes. It delivers radiation doses at an ultra-high dose rate (UHDR: ≥ 40
20 Gy/s) in a single instant or a few pulses. Much higher irradiation doses can be
21 administered to tumors with FLASH-RT than with conventional dose rate
22 (0.01–0.40 Gy/s) radiotherapy. UHDR irradiation can suppress toxicity in
23 normal tissues while sustaining antitumor efficiency, which is referred to as
24 the FLASH effect. However, the mechanisms underlying the effects of the
25 FLASH remain unclear. To clarify these mechanisms, the development of
26 simulation models that can contribute to treatment planning for FLASH-RT
27 is still underway. Previous studies indicated that transient oxygen depletion
28 or augmented reactions between secondary reactive species produced by
29 irradiation may be involved in this process. To discuss the possible
30 mechanisms of the FLASH effect and its clinical potential, we summarized
31 the physicochemical, chemical, and biological perspectives as well as the
32 development of simulation modeling for FLASH-RT.

33

34 **Keywords:** FLASH radiotherapy, reactive species, simulation modeling,
35 ultra-high dose rate

36 **Abbreviations**

37 BED biologically effective dose

38 CONV conventional

39 DSB double-strand break

40 GSH glutathione

41 IRT independent reaction time

42 LET linear energy transfer

43 LPO lipid peroxidation

44 LQ linear-quadratic

45 MD molecular dynamics

46 OER oxygen enhancement ratio

47 ROS reactive oxygen species

48 SBS step-by-step

49 SSB single-strand break

50 TOD transient oxygen depletion

51 UHDR ultra-high dose rate

52

53 **1. Introduction**

54 Since the late 1950s, the unique biological effects of ultrahigh dose rate
55 (UHDR) irradiation have been investigated (Fig. 1) [1–5]. In 1958, Kirby-
56 Smith and Dolphin were the first to demonstrate decreased chromosomal
57 breakage in *Tradescantia* microspores and pollen at high dose rates (1.0 and
58 4.0×10^6 Gy/s) compared to low dose rates (≤ 0.01 Gy/s). They proposed
59 two concepts to interpret these results: recombination of organic free radicals
60 and partial temporal anoxia caused by the immediate consumption of oxygen
61 [1]. In the following year, Dewey and Boag discovered an increase in the
62 surviving fraction of *Serratia marcescens* with UHDRs under hypoxic
63 conditions and suggested that the effects may have been caused by the
64 removal of dissolved oxygen [2].

65 Recent preclinical studies revealed considerable tissue-sparing
66 responses following UHDR irradiation [4,6–15], including reduced
67 pulmonary fibrosis and cutaneous lesions [6], extended survival times [6],
68 lower skin toxicity [7], fewer gastrointestinal symptoms [8], and preserved
69 cognitive function [9,10]. Meanwhile, in some *in vivo* studies [4,6–
70 8,10,16,17], the equivalence of antitumor efficacy at the UHDR to the
71 conventional (CONV) dose rate has been indicated using several models (i.e.,

72 orthotopic lung carcinoma [6], squamous cell carcinoma [7], metastatic
73 ovarian cancer [8], orthotopic striatum glioblastoma [10], human pharyngeal
74 carcinoma xenografts [16], and pancreatic tumors [17]). These *in vivo* studies
75 were performed using low linear energy transfer (LET) radiation (i.e.,
76 electrons, X-rays, and protons). Some studies using protons showed a larger
77 decrease in lung tumor proliferation at UHDR [18,19]; however, others
78 showed tumor growth inhibition equivalent to that under the CONV dose rate
79 [16,17]. One experiment utilizing the plateau region of the Bragg curve of
80 carbon ions (higher LET than protons but lower than that in the Bragg peak
81 of carbon ions) at UHDR indicated sparing of normal tissue with diminishing
82 growth and metastasis of osteosarcoma compared to the CONV dose rate [20].
83 Accumulation of knowledge from UHDR research has led to the development
84 of an innovative radiation treatment called FLASH radiotherapy (FLASH-
85 RT). In the novel FLASH-RT technique, radiation doses are delivered using
86 higher dose rates (i.e., ≥ 40 Gy/s) than in CONV radiotherapy (CONV-RT)
87 (i.e., 0.01–0.40 Gy/s). The considerable sparing effect at UHDR both *in vivo*
88 and *in vitro* has been observed when using a high dose irradiation (≥ 10 Gy)
89 [21,22]. Focusing on *in vivo*, the sparing effect depends on the relationship
90 between the dose rate per pulse and total irradiation time[10]. Therefore, not

91 only the dose rate, but also the dose per fraction and pulse structure are
92 important when evaluating the sparing effect in FLASH-RT.

93 The most important feature of FLASH-RT is its superior ability to spare
94 normal tissues compared to CONV-RT while preserving antitumor efficacy,
95 which is known as the FLASH effect. FLASH-RT has the potential to improve
96 the limited tumor control of CONV-RT by increasing the prescribed dose for
97 tumors by virtue of the possibility of a normal tissue-sparing effect. The first
98 in-human study [23] and clinical trial [24] on UHDR irradiation demonstrated
99 this potential. However, the reasonable prescribed dose for the FLASH effect
100 remains unclear. While previous studies have compared the early toxicity
101 between FLASH-RT and CONV-RT, some have investigated late toxicity.
102 Gaide *et al.* indicated that both FLASH-RT and CONV-RT, with a single dose
103 of 15 Gy targeting one patient, resulted in the same degree of early and late
104 toxicity [25]. Bley *et al.* indicated that single-dose irradiation with 30 Gy in
105 FLASH-RT induced more severe late toxicity compared to 4.8 Gy
106 hypofractionated irradiation in CONV-RT [26]. It should be noted that the
107 latter toxicity was observed using the different biologically effective dose
108 (BED) values [26]. Considering this, a precise assessment of the threshold
109 dose that can spare the late toxicity of FLASH compared with CONV is

110 essential.

111 Recently, several studies have proposed potential factors involved in
112 FLASH mechanisms after UHDR exposure, such as transient oxygen
113 depletion (TOD) and augmented reactions between secondary reactive
114 species produced by irradiation. In this review, to discuss the potential
115 mechanisms of the FLASH effect and future clinical applications, we
116 summarized the literature, mainly focusing on the physicochemical, chemical,
117 and biological stages, as well as existing simulation models for FLASH-RT.
118 In this review, we discuss the current state of experimental and theoretical
119 studies, as well as future prospects.

120

121 **2. Hypothetical physicochemical and chemical mechanisms**

122 **2.1. Transient oxygen depletion hypothesis**

123 Previous studies have suggested that oxygen depletion may be a critical factor
124 in the sparing response to UHDR irradiation [1–5]. Oxygen depletion is
125 primarily caused by the immediate reactions of dissolved molecular oxygen
126 with radiation-synthesized hydrated electrons (e_{aq}^-) and hydrogen atoms ($H^\bullet$)
127 [2,12,27,28]. High oxygen consumption during FLASH-RT has been
128 interpreted as diminishing biological effects through the attenuation of

129 oxygen effects (i.e., reducing the oxygen enhancement ratio (OER)).
130 Particularly in hypoxic environments, the reduction in oxygen concentration
131 could induce a further sparing effect as the OER dramatically changes. A
132 recent report showed that the surviving fraction of the prostate cancer cells
133 DU145 irradiated under normoxic conditions showed a non-significant
134 difference between the FLASH and CONV dose rates, whereas hypoxia
135 induced a substantial difference [29]. Anoxic conditions do not induce sparing
136 effects at the FLASH dose rate [1,30,31].

137 However, the transient oxygen depletion (TOD) hypothesis has been
138 questioned in recent years [13,14,22,32]. This sparing effect has been
139 experimentally observed in the oxygen-rich lungs [6] and brains [9] of mice
140 and in some cancer cell lines under normoxia [33]. Measurements of oxygen
141 pressure have also showed that irradiation at the UHDR consumes less
142 oxygen than exposure to CONV *in vitro* [34] and in water [35]. *In vivo* studies
143 have indicated insufficient oxygen consumption for the sparing effect [34,36].
144 These suggested that the aforementioned e_{aq}^- and $H\cdot$ may be largely
145 consumed through self-annihilation before they interact with dissolved
146 oxygen at FLASH dose rate [35]. However, it should be noted that the
147 maximal time resolutions of their oxygen sensors (~150 ms for the OxyLED

148 phosphorometer [Oxygen Enterprises Ltd.] [34] and ~400 ms for FireStingO2
149 [PyroScience GmbH] [35]) are longer than the exposure durations of FLASH-
150 RT (a few nanoseconds to 100 ms) and the 10–20 ms duration in which cells
151 are no longer susceptible to local oxygen concentration [37,38]. Therefore,
152 the instantaneous oxygen depletion may not have been adequately observed
153 [34,35].

154 Meanwhile, the high-LET radiation in the Bragg peak regions, such as
155 carbon ions, has been considered to reinforce oxygen generation at the UHDR
156 [39,40]. The increase in oxygen production was suggested to be elicited by
157 the highly dense reactive species synthesized via multiple ionization reactions
158 [39].

159

160 **2.2. Self-detoxification of reactive species**

161 Another hypothesis for interpreting the sparing effect of FLASH-RT is the
162 consumption of reactive species via inter-track interactions [41–45]. A cluster
163 of a few reactive species in the vicinity of the ionization and excitation sites
164 is called a spur [46,47]. Although the spatial distribution of spurs under
165 FLASH-RT should be similar to that under CONV-RT during the entire
166 irradiation time, the distribution per unit time under FLASH-RT is transiently

167 much denser than that under CONV-RT because a substantial number of
168 tracks arrive almost concurrently at the UHDR. Such spatiotemporally dense
169 species may promote interactions between the reactive species generated in
170 different spurs, which can be attributed to different tracks (intertrack
171 interactions). Inter-track interactions may result in the transformation of
172 detrimental species into inert species before these species oxidize
173 biomolecules such as DNA and membranes. Some experimental studies have
174 estimated that high dose rates can reduce the yields of hydrogen peroxide
175 (H_2O_2) [9,15,48] and hydroxyl radicals ($\cdot\text{OH}$) [49,50], suggesting that
176 intertrack interactions diminish deleterious species in FLASH-RT. Notably
177 these yields of H_2O_2 and $\cdot\text{OH}$ were indirectly measured using the interactions
178 between each species and the corresponding reagents in water [48–50].

179

180 **3. Hypothetical biological mechanisms**

181 **3.1. DNA damage and cell survival fraction**

182 Considering the reduced yield of $\cdot\text{OH}$ under UHDR conditions compared to
183 that under CONV conditions [49,50], the yield of DNA double-strand breaks
184 (DSBs), which are intrinsically related to cell death with a certain probability,
185 under FLASH irradiation can be smaller than that under CONV irradiation.

186 Some studies using normal cells have demonstrated lower DSB yields with
187 FLASH than with CONV. Levy *et al.* showed that the yield of γ -H2AX foci
188 (one of a DSB marker produced by the phosphorylation of histone H2AX near
189 a DSB site) in crypt-based columnar (CBC) cells were smaller after UHDR
190 irradiation with 14 Gy compared to CONV irradiation [8]. Fouillade *et al.*
191 found a non-significant difference in the number of γ -H2AX in normal human
192 lung fibroblast cells (MRC5 and IMR-90) after irradiations with 5.2 Gy at
193 FLASH dose rate compared to CONV dose rate [51]. Meanwhile, the number
194 of foci of 53BP1 (another DSB marker that can be recruited to γ -H2AX for
195 the DSB repair) was reduced in same cell lines (i.e., MRC5 and IMR-90).
196 From these results for γ -H2AX and 53BP1, Fouillade and colleagues
197 suggested that the DSB yields are almost independent of the dose rate,
198 whereas the FLASH irradiation inhibits 53BP1 to recognize a subset of DNA
199 damage [51] (e.g., dimethylated histone H4 Lys 20 (H4K20me2) and H2A
200 ubiquitinated on Lys 15 (H2AK15ub) which are recognized by 53BP1 and
201 accumulate 53BP1 at the DSB sites [52]) compared to CONV dose rate.
202 Conversely, the unaltered yield of γ -H2AX in IMR-90 cells with 5.2 Gy is
203 consistent with another result indicating that only ≥ 20 Gy doses induce
204 difference of those between FLASH and CONV irradiations [53]. Hence, a

205 significant difference in DSB yields between the FLASH and CONV dose
206 rates can be observed when irradiating the cells at high doses. Additionally, it
207 has been proposed that the subsequent DSB repair capacity may be
208 diminished, even in the case of low doses of UHDR, by 53BP1 reduction [51].
209 Therefore, FLASH irradiation can increase the levels of apoptosis inducers,
210 such as cytochrome C and cleaved caspase-3, compared with the CONV dose
211 rates [54]. CONV irradiation was associated with enhanced cell death and a
212 much higher fraction of necrotic cell death than that of apoptosis [54].

213 Studies using tumor cells, such as the mouse ovarian surface epithelial
214 cell line ID8, revealed no significant differences in the number of γ -H2AX
215 foci in contrast to the above-stated normal CBC cells treated with the same
216 beam parameters except for dose rate [8]. Similarly, the 53BP1 yields in
217 tumor cells in the human lung adenocarcinoma cell line A549 irradiated at the
218 FLASH dose rate were at the same level as those at the CONV dose rate
219 despite the altered 53BP1 yields in normal cells (i.e., MRC5 and IMR-90)
220 treated with the same beam parameters other than dose rate [51]. Adrian *et al.*
221 also showed an equivalent number of 53BP1 foci in the human squamous cell
222 carcinoma cell line LU-HNSCC4, human breast cancer cell line MDA-MB-
223 231, and human cervical cancer cell line HeLa between FLASH and CONV

224 irradiation [33]. Meanwhile, the measured surviving fraction increased
225 slightly or substantially under FLASH irradiation [33]. Based on limited
226 information on tumor cells, the equivalent DSB induction and repair capacity
227 between FLASH and CONV irradiation is consistent with the *in vivo*
228 sustained antitumor efficacy, whereas the cell-surviving fraction may not be
229 largely associated with the *in vitro* DSB yield and *in vivo* results. Moreover,
230 sustained DSB yield and repair pathway expression in tumor cells cannot be
231 explained by the aforementioned difference in the yield of toxic reactive
232 species observed in water between FLASH and CONV irradiation. The
233 tumor-specific redox balance may be partly related to the unaltered DSB
234 induction between FLASH and CONV irradiation. The levels of reactive
235 species in tumor cells are higher than those in normal cells, and tumor cells
236 heavily develop antioxidant systems, such as glutathione (GSH) peroxidase
237 and catalase, to regulate the toxicity of reactive species [55–59]. Furthermore,
238 tumor cells are presumed to be more vulnerable to additional reactive species
239 and the inhibition of antioxidant systems than normal cells because the
240 original level of reactive species in tumor cells is close to the cell death
241 threshold [55–59]. Given that radiation can increase reactive species and
242 restrain GSH content [59], tumor cells may be killed independently of the

243 dose rate because both FLASH and CONV irradiation may produce sufficient
244 reactive species, combined with adequate suppression of antioxidant systems,
245 to accomplish the death threshold for the concentration of reactive species.

246 In an experiment with pBR322 plasmid DNA comparing FLASH and
247 CONV irradiation, researchers reported a reduction in the number of DNA
248 single-strand breaks (SSBs), but no significant differences in the number of
249 DSBs under FLASH irradiation [60]. SSBs may influence the sparing effect
250 at the FLASH dose rate (such as the conversion of SSBs into single-ended
251 DSBs during the S phase [61–63]).

252 Based on the previous experimental reports, for normal tissues, the DSB
253 yield [8,51,53] and sequential repair process [51] may not necessarily largely
254 contribute to the consequent FLASH effect [22]. Those in tumor cells that
255 were not altered between FLASH and CONV irradiation [8,33,51] were not
256 in conflict with the conserved antitumor efficacy. However, LU-HNSCC4,
257 MDA-MB-231, and HeLa tumor cells irradiated with UHDR showed a slight
258 or considerably increase in surviving fractions despite similar DSB
259 expression levels between FLASH and CONV irradiation [33]. In addition,
260 the cell surviving fraction of human salivary gland tumor cells HSGc-C5 and
261 normal human fibroblasts HFL1 was not altered after UHDR-carbon ion

262 irradiation in the plateau region and vicinity of the Bragg peak between
263 FLASH and CONV irradiation [64], which contradicts the aforementioned *in*
264 *vivo* results [20]. To comprehend the inconsistency between the results of *in*
265 *vivo* and *in vitro*, further accumulation of experimental data, consideration of
266 experimental conditions (e.g., spheroids and organoids), [32] and other
267 factors (e.g., lipids and proteins in the cytoskeleton [22] and mitochondria
268 [54]) are essential. For instance, in an approach with spheroids (an ideal
269 model to investigate UHDR-elicited oxygen depletion within tumor tissues
270 owing to the uneven oxygen distribution (i.e., hypoxic core) within spheroids),
271 the experiment showed that the survival fraction of A549 tumor cells
272 constituting spheroids under normoxic conditions and UHDR irradiation was
273 considerably higher than that under CONV dose-rate irradiation, while
274 demonstrating no differences in cell cultures [65]. Spheroids resulted in a
275 hockeystick survival curve that differed from the linear-quadratic (LQ) shape
276 [65], which has long been believed to be attributed to transient anoxia induced
277 by the combination of UHDR and high dose [3,21,65]. However, these results
278 contradict the *in vivo*-sustained antitumor efficacy, which was attributed to a
279 lack of vasculature and immunogenic cell death in a spheroid [65]. The
280 balance between the hypoxic core and the vasculature may result in *in vivo*

281 preserved antitumor efficacy.

282

283 **3.2. Lipid peroxidation and ferroptosis**

284 Lipid peroxidation (LPO) and ferroptosis (a form of regulated cell death that

285 is morphologically and mechanistically distinct from apoptosis, which is the

286 most well-known type of regulated cell death [66,67]) have recently attracted

287 attention as promoters of the sparing effect of FLASH-RT [28,68,69].

288 Ferroptosis is thought to be attributed to the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow$

289 $\cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$) and the accumulation of lipid peroxides synthesized by

290 the generated $\cdot\text{OH}$ [68–70]. Radiation-induced $\cdot\text{OH}$ oxidizes membranes

291 containing polyunsaturated fatty acids, resulting in LPO and consequently,

292 ferroptosis [70–72]. An investigation utilizing phosphatidylcholine liposomes

293 and linoleic acid micelles as proxies for cellular membranes demonstrated

294 that FLASH irradiation restricts LPO more effectively than CONV irradiation

295 [69], indicating that FLASH-RT may suppress ferroptotic cell death.

296

297 **4. Simulation models for FLASH-RT**

298 Many simulation models for the FLASH-RT have been developed to clarify

299 the mechanisms of the FLASH effect. These models are useful for supporting

300 treatment planning for FLASH-RT and proposing possible mechanisms.
301 Herein, we discuss the current status of the development of simulation models.
302 Tables 1 and 2 list the estimation frameworks based on Monte Carlo
303 simulation [39,41–45,73–79] and other theoretical models [36,37,45,65,80–
304 90], respectively.

305

306 **4.1. Monte Carlo track structure simulations**

307 Monte Carlo simulation was used to analyze the track structures of the
308 incident particles and chemical products resulting from water radiolysis. To
309 date, various Monte Carlo codes (PARTRACK [41], TOPAS-nBio
310 [42,44,45,77], IONLYS-IRT [39,43], gMicroMC [74], NASIC [75,76],
311 Geant4-DNA [78,79], and TRAX-CHEM [73]) have been presented and
312 applied to FLASH studies to investigate FLASH mechanisms. Focusing on
313 the chemical processes that are important in the FLASH study, the initial
314 yields of chemical products (the so-called G values at 1 ps) can be predicted
315 using a physicochemical model (the so-called branching model) based on
316 physical processes (i.e., atomic interaction types). In the latter phase, that is
317 chemical diffusion, reactions, and generation, there are two major simulation
318 methods. One is the step-by-step (SBS) approach, [41,44,45,73–75] which is

319 time-consuming, but enables control of the spatial distribution of all species
320 at all times. The second is the independent reaction time (IRT) approach,
321 which can reduce calculation costs without tracking the trajectories of the
322 species [39,42,43,76]. Additionally, coupled nonlinear ordinary differential
323 equations [77] and molecular dynamics (MD) simulations [78,79] are used in
324 chemical studies worldwide.

325 In general, Monte Carlo simulation studies made several
326 assumptions for the FLASH effect: (i) transient oxygen depletion (TOD) [73–
327 76,78], (ii) inter-track interaction [41–45,79], (iii) radical-radical
328 recombination at long time scales [77], (iv) screening effect by the chains of
329 complexes consisting of reactive species [78], (v) augmented diffusion of
330 reactive species via influx of thermal energy from the surrounding excited
331 molecules [79] in low-LET irradiation, and (vi) reinforcement of oxygenation
332 in high-LET irradiation [39]. The simulation results following assumption (i)
333 showed insufficient oxygen depletion [73–75], which is consistent with the
334 measured oxygen pressure [34–36], suggesting that the sparing effects under
335 both *in vivo* and *in vitro* in physoxic conditions [6,9,33] cannot be induced
336 via oxygen depletion. In the following sections, we summarize the
337 interpretations based on assumptions (ii)–(vi) (see Section 4.1.1, for

assumptions (ii), (iii), and (vi), and Section 4.1.2, for assumptions (iv) and (v)). It is needed to recognize the following typical assumptions and limitations of the Monte Carlo chemical simulation codes: (A) radiation irradiation toward pure liquid water instead of the scavenging conditions in cells that can result in unprecise estimation of the behavior of reactive species within a cell/tissue, and (B) restricted time scale to the range of the physicochemical to chemical stage (1 ps–1 μ s) because of the large computational time. Focusing on assumption (A), Baikalov *et al.* simulated G values under cellular conditions [45] for UHDR irradiation (see Section 4.1.1). Regarding limitation (B), the TOPAS-nBio [42] and IONLYS-IRT [43] codes for UHDR using the IRT method enabled longer-timescale simulations (up to 10 ms and 1 ms, respectively). However, IRT is not suitable for investigating FLASH mechanisms because it does not track the trajectories of chemical species [42,43]. Another code, TRAX-CHEMxt, can be further developed to study the FLASH-sparing effect [91].

4.1.1. Inter-track interactions under UHDR irradiation

Inter-track interactions under UHDR might frequently convert deleterious reactive species (such as $\cdot\text{OH}$ and e_{aq}^-) to relatively inert products (such as

357 H₂O and H₂). Calculations using Monte Carlo codes considering intertrack
 358 interactions suggested that dose rate escalation could reduce the yields of $\cdot\text{OH}$
 359 in pure water, which is consistent with the experiments that demonstrated
 360 diminished $\cdot\text{OH}$ yields at UHDR [49,50]. However, the calculated increase in
 361 H₂O₂ production following the dose-rate increment [41,43,44] contradicts the
 362 experimental data that demonstrate the suppression of H₂O₂ generation at the
 363 UHDR[9,15,48]. The H₂O₂ yields could be mainly determined by the reaction
 364 $\cdot\text{OH} + \cdot\text{OH} \rightarrow \text{H}_2\text{O}_2$ [45]. Meanwhile, it is believed that the reaction $\cdot\text{OH} +$
 365 $\text{e}_{\text{aq}}^- \rightarrow \text{OH}^-$ could be enhanced by inter-track interaction; that is, the yield of
 366 H₂O₂ could be suppressed at the FLASH dose rate. This may be a reasonable
 367 mechanism to explain the experimental chemical yields [9,15,48]. Based on
 368 a Monte Carlo simulation with the IRT method considering clinically relevant
 369 pulse lengths (i.e., 1 ns, 1 μs , and 10 μs) when delivering 1 and 100 MeV
 370 protons with 50 cGy to water, the simulation results showed that the yields of
 371 $\cdot\text{OH}$ and e_{aq}^- are largely reduced after irradiation with 100 MeV protons at
 372 UHDR, whereas 1 MeV protons resulted in smaller suppressions of $\cdot\text{OH}$ and
 373 e_{aq}^- generations [42]. This finding suggests that intertrack interactions are
 374 important, leading to significant FLASH-sparing effects for low-LET
 375 radiation [42] from a chemical viewpoint. While most of the inter-track

376 simulations were performed in liquid water [41–44], Baikalov and colleagues
377 considered the half-lives of each species (i.e., $\cdot\text{OH}$, e_{aq}^- , $\text{H}\cdot$, and H_2O_2 , that
378 can induce biological lesions) and investigated the impacts of the inter-track
379 interaction on the chemical reactions under $\cdot\text{OH}$ scavenging condition in cells
380 with the SBS method [45]. The results showed no differences in the yields of
381 the chemical species between FLASH and CONV in the cell condition even
382 after 58 Gy electron irradiation while demonstrating the significant
383 suppression of the yields of part of the species ($\cdot\text{OH}$ and e_{aq}^-) at FLASH in
384 physoxic (4% O_2) water [45]. Further studies of the cellular environment and
385 accumulation of experimental data for incorporation into such simulations are
386 required to clarify the mechanisms of sparing effects based on inter-track
387 interactions. However, it should be noted that the migration distance of the
388 reactive species synthesized at the FLASH dose rate may be prolonged, as the
389 authors discussed [45] based on the report by Abolfath *et al.* [79] (see Section
390 4.1.2).

391 A Monte Carlo simulation considering the inter-track interaction for
392 high-LET carbon ions using IONLYS-IRT showed oxygenation in the Bragg
393 peak region due to multiple ionizations, despite non-significant oxygenation
394 in the plateau region[39]. The measurement of oxygen pressure within a water

phantom after FLASH irradiation of 10 Gy dose with carbon ions at the plateau region showed a slight decrease in oxygen consumption with dose rate escalation for 150 MeV/u (19.47 keV/ μ m) carbon ions while indicating no changes for 400 MeV/u (10.89 keV/ μ m)[35]. Hence, the LET increment in the plateau region induces a dose rate-dependent reduction in oxygen consumption, which might be due to oxygenation, as estimated in the *in silico* model[39]. However, oxygen consumption/replenishment measurements were not performed in the Bragg peak region [20,35,64]. Thus, it remains unclear whether the carbon ions in the Bragg peak region can oxygenate cells, similar to the calculations [39].

405

4.1.2. Combination with molecular dynamics simulation

Molecular dynamics (MD) simulation is a powerful tool that enables the estimation of physicochemical and chemical processes by analyzing molecular statistical properties such as geometrical structures, energy evolution, and dipole moment evolution [92]. Recent approaches have combined Monte Carlo and MD simulations [78,79]. In the first study, it was suggested that hydrogen abduction in DNA after proton irradiation at the UHDR could be delayed because highly dense reactive oxygen species (ROS)

414 including $\cdot\text{OH}$, significantly suppress their oxidative effects. In addition, ROS
415 can form nonreactive chain-like clusters through attractive electric polarity
416 forces, hydrogen bonds, and magnetic dipole-dipole interactions [78]. In the
417 second study, thermal energy transfer to reactive species from surrounding
418 excited molecules through the phonon field under FLASH irradiation was
419 estimated, which resulted in an escalation of the diffusion coefficients of the
420 reactive species [79]. While the cellular conditions may inhibit inter-track
421 interactions in the range of clinically adoptable doses, such augmented
422 diffusion coefficients may facilitate inter-track interactions [45].

423 Calculations using these two simulation models presented contrasting
424 alterations in the diffusion coefficients (the former proposed a smaller value
425 and the latter a larger value). Although the degree of contribution of molecular
426 dynamics to the true alteration in the behavior of the reactive species remains
427 unclear, it is important to consider such molecular dynamics when
428 interpreting the difference in reactive species mobility between FLASH and
429 CONV and the consequent FLASH effect.

430

431 **4.2. Other theoretical models**

432 To date, several researchers have made the following assumptions to interpret

433 the mechanism of the FLASH effects: (a) oxygen depletion[36,80–88], (b)
434 intertrack interaction [45], (c) reactions between reactive species and
435 biomolecules [37], and (d) preservation of circulating immune cells[89,90]
436 (listed in Table 2). Although most of these studies have focused on low-LET
437 radiation (LET values are not specified in each report), few models have
438 considered LET dependence in the low-LET range [45,88]. These simulations
439 assume high single doses (≥ 10 Gy) based on both *in vitro* and *in vivo* results
440 [21,22].

441 Although numerous models considering oxygen kinetics have been
442 proposed [36,37,65,80–88], some predict insufficient oxygen consumption
443 for the sparing effect [36,37,80,82,84], which is consistent with several
444 studies questioning the TOD hypothesis [13,14,22,32] (see Section 2.1) and
445 Monte Carlo simulations [73–75]. The inapplicability of a dose that can
446 induce oxygen depletion in physoxia for clinical use was also suggested, i.e.,
447 a 71.4 Gy dose per pulse with 71.4 Gy/s dose rate for 3.9% physoxic
448 conditions [36]. In addition, a model accounting for the heterogeneity of the
449 oxygen partial pressure within tissues [93] was proposed [86]. In this model,
450 it was assumed that inhomogeneity in the local partial oxygen pressure may
451 induce the FLASH-sparing effect. Oxygen diffusion, metabolism, and

452 radiolytic oxygen depletion were simulated using randomly positioned
453 oxygenated capillary vessels at doses of 20 Gy. The results indicated that the
454 sparing effect depended on the extended localized hypoxic or anoxic areas
455 after FLASH irradiation, whereas the decrease in the mean partial oxygen
456 pressure was inadequate for sufficient suppression of oxygen effects in
457 normal tissues. However, the hypoxic regions cannot be dramatically
458 increased in previously hypoxic tumor tissues, even at UHDR, which implies
459 sustained antitumor efficacy. Another approach for spheroids, considering the
460 dynamics of oxygen concentration, estimated TOD via the hypoxic core and
461 reproduced the hockey-stick survival curve [65]. Although the relevance of
462 this model to *in vivo* tumor models has not been clarified, it has been implied
463 that tumor cells, especially those exposed to hypoxic conditions within tumor
464 tissues, could be spared via oxygen depletion at high doses of FLASH [65].

465 A theoretical model, considering various interactions between reactive
466 species and biomolecules after the nonhomogeneous chemical stage (i.e., $\geq$
467 1 μ s), predicted a decrease in the accumulated yield of detrimental peroxy
468 radical ROO $\cdot$ with the escalation of dose rate [37]. It was also estimated that
469 the higher the delivered radiation dose, the more dependent the accumulated
470 yield of ROO $\cdot$ was on the dose rate. In addition, insufficient oxygen depletion

471 was calculated for the FLASH-sparing effect.

472 The most recent theoretical model, assuming cellular conditions,
473 presents the dose and pulse width necessary for inducing intertrack
474 interactions [45]. Importantly, this model predicted that a radiation dose > 90
475 Gy per 0.5 μ s pulse might be needed for sufficient inter-track interaction to
476 affect the yields of reactive species in a cell with $\cdot$ OH scavenging. Meanwhile,
477 the model estimated a dose > 13 Gy for 4% physoxic water, implying the
478 significance of further studies that consider the scavenging capacity of cells.
479 As discussed in the report by Baikalov *et al.* [45], it is also essential to note
480 that UHDR irradiation may prolong the diffusion range of reactive species
481 (see Section 4.1.2), which could induce intertrack interactions.

482 A model that considers the effects of radiation on circulating immune
483 cells was proposed [89,90]. This model considers the blood circulation system
484 of the heart and numerous blood-containing organs with parallel and/or serial
485 connections. In addition, in the simulation setup, the dependence of the
486 irradiation time on the dose rate, irradiated and unirradiated areas, and blood
487 circulation time were explicitly considered. The model predicted that
488 FLASH-RT would result in a substantially higher preservation of circulating
489 immune cells than CONV-RT, which is consistent with the experimental

490 results [93]. It has also been suggested that a higher prescription dose per
491 fraction could spare more immune cells with dose rate escalation. Although
492 unirradiated immune cells can flow into irradiated areas within a relatively
493 long radiation delivery time in CONV-RT, the additional influx of unexposed
494 cells may be considerably limited in FLASH-RT owing to the transient
495 irradiation time. These spared immune cells may enhance radioresistance in
496 normal cells, because the immune system is recruited to repair radiation-
497 induced lesions [89]. The conserved immune system may improve tumor
498 control probability, as lymphopenia results in poor tumor control and shorter
499 survival times [18,94].

500

501 **4.3. Current state and prospects**

502 Most simulation studies have investigated the mechanisms underlying the
503 effects of FLASH (i.e., the normal tissue-sparing effect and sustained
504 antitumor efficacy). Although the detailed mechanisms remain uncertain from
505 an experimental viewpoint, several models have been proposed that allow the
506 prediction of radiation effects in liquid water based on physical, chemical,
507 and biological processes. Simulation models for the physical and chemical
508 stages (such as intertrack interactions [41–45,79], radical-radical

509 recombination [37,77], and inactivation of ROS [78]) have suggested the
510 possibility of reducing cellular damage (e.g., DNA lesions) during FLASH
511 irradiation. Meanwhile, focusing on biological processes based on the
512 physical and chemical stages, mechanistic simulations for directly analyzing
513 the effects of FLASH based on reactive species yields (chemical reactions)
514 do not exist. Therefore, the development of a model that enables the
515 connection between reactive species yields and biomolecule lesions (e.g.,
516 type and complexity) in heterogeneous cell populations (composed of normal,
517 tumor, and immune cells) for various tissue-specific environments (e.g.,
518 reactive species, labile iron Fe^{2+} , and antioxidants [28,55–59]) would
519 contribute to the clarification of FLASH effects in the future.

520 In the current state, the previous simulation models for FLASH-RT have
521 been proposed based on both a limited amount of experimental data and the
522 model assumptions without any observations. To develop a detailed
523 mechanistic simulation model for FLASH-RT, the accumulation of
524 experimental data that can connect the physical, chemical, and biological
525 processes is mandatory. Therefore, in future FLASH studies, biological
526 experiments to identify the dominant modification types of cellular lesions
527 (e.g., reactions to lipids, proteins, and mitochondria, in addition to DNA

528 [22,54,69]) regarding physical and chemical processes are urgently needed.

529

530 **5. Conclusion**

531 The efficacy of FLASH is regulated by various factors occurring in an

532 extensive range of physicochemical and biological phases after irradiation.

533 However, the mechanisms underlying the effects of the FLASH remain

534 unclear. Interestingly, the estimated increase in the production of H_2O_2

535 following the dose-rate increment contradicted experiments that suggested

536 the suppression of H_2O_2 generation under FLASH irradiation. Thus, further

537 interdisciplinary investigations in radiation physics, chemistry, biology, and

538 oncology are required to clarify the underlying mechanisms of the FLASH

539 effect and to develop simulation models for FLASH-RT treatment planning.

540

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544

545 **Statements and Declarations**

546 *Conflicts of interest/Competing interests:* The authors declare no conflicts of

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550

551

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876 **Tables**

877 Table 1. Details of Monte Carlo simulations for UHDR. Monte Carlo

878 simulation code, target duration for simulation, radiation type, target

879 hypothesis for the FLASH-sparing effect, and output were listed.

Monte Carlo simulation code	Target duration	Radiation type	Target hypothesis	Output	Reference
TRAX-CHEM	$\sim 1 \mu\text{s}$	Electrons	Transient oxygen depletion (TOD)	• Insufficient removal of oxygen to support oxygen depletion hypothesis	Boscolo et al. 2021 [73]
gMicroMC	$\sim 1 \mu\text{s}$	Protons	TOD	• Insufficient oxygen depletion at any initial oxygen concentration (0.01- 21%) with a 30 Gy cumulative dose	Lai et al. 2021 [74]
NASIC	$\sim 1 \mu\text{s}$	• Electrons • protons	TOD	• Oxygen depletion only in hypoxic condition • Larger transient oxygen depletion with lower LET • Decreased DNA damage and increased survival fraction under only hypoxic condition	Zhu et al. 2021 [75]
NASIC	$\sim \text{several}$ μs	Electrons	TOD	• Dependence of oxygen depletion on the initial oxygen concentration and the diffusion of oxygen	Hu et al. 2022 [76]
PARTRAC	$\sim 1 \mu\text{s}$	• Protons • He^{2+} • C^{6+}	Inter-track interaction	• Reduction yields of $\cdot\text{OH}$ and e_{aq}^- for UHDR- He^{2+} • Increased yields of H_2O_2 and H_2 for UHDR-5 MeV/u C^{6+} • Suppressed yield of clustered lesions for protons and He^{2+} • Suppressed yield of DSB for UHDR- He^{2+}	Kreipl and Friedl 2009 [41]

TOPAS-nBio	~ 10 ms	Protons	Inter-track interaction	• Decreased yield of $\cdot\text{OH}$ and e_{aq}^- under 100 MeV proton irradiation and slight reduction in $\cdot\text{OH}$ and e_{aq}^- yields under 1 MeV proton irradiation at FLASH	Ramos-Méndez et al. 2020 [42]
IONLYS-IRT	~ 1 ms	Protons	Inter-track interaction	• Reduced yields of $\cdot\text{OH}$ and e_{aq}^- and increased yields of H_2O_2, H_2, and $\text{HO}_2^\cdot/\text{O}_2^{\cdot-}$ at FLASH • The pronounced maximum decrease in O_2 yield at 0.2 μs after irradiation with dose rate increase	Alanazi et al. 2021 [43]
TOPAS-nBio	~ 1 μs	Protons	Inter-track interaction	• Decreased yields of $\cdot\text{OH}$, e_{aq}^-, and H_3O^+ at FLASH • Slightly increased yields of OH^-, H_2O_2, and H_2 at FLASH • Requirement of lower dose to saturate $\cdot\text{OH}$ yield with LET increase	Derksen et al. 2023 [44]
TOPAS-nBio	~ 1 μs	Electrons	Inter-track interaction	• No difference in yields of $\cdot\text{OH}$, e_{aq}^-, H_2O_2, and $\text{H}^\cdot$ in cellular scavenging conditions after 58 Gy delivery • Decrease in yields of $\cdot\text{OH}$ and e_{aq}^- in physoxic water after 58 Gy delivery • Dependence of the expression of the sparing effect on the pulse dose and pulse width	Baikalov et al. 2023 [45]
TOPAS-nBio	~ 1 μs	Protons	• Radical-radical recombination • Reactions among reactive species, biomolecules, and antioxidants	• Decrease in the synthesis of $\text{ROO}^\cdot$ (surrogate for biological damage) with the increase in the mean dose rate • Dependence of the steady state of $\text{ROO}^\cdot$ on the pulse frequency and the dose per pulse under the fixed total dose and mean dose rate	Espinosa-Rodriguez et al. 2022 [77]

Geant4-DNA	~ 1 ns	Protons	Screen effect by the chains of complexes consist of reactive species (NROS)	• Larger ROS production ($\cdot\text{OH}$, H_2O_2, singlet oxygen, O_2^-, and alpha-oxygen are added up together as ROS) at UHDR • Larger ROS consumption via the construction of NROS	Abolfath et al. 2020 [78]
Geant4-DNA	~ 1 ns	Protons	Influx of thermal energy to reactive species from the surrounding excited molecules	• Increased diffusion coefficient of reactive species through the influx of thermal energy from the surrounding excited species	Abolfath et al. 2022 [79]
IONLYS-IRT	~1 μs	Carbon ions	Multiple-ionization	• Escalated O_2 generation at the Bragg peak • No increase in O_2 production at the plateau region	Zakaria et al. 2020 [39]

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881

882 Table 2. Summary of theoretical models. Model overview, target hypothesis

883 for the FLASH-sparing effect, and output were listed.

Model Overview	Target hypothesis	Output	Reference
Differential equations considering diffusion from capillary, consumption, and radiolytic depletion of oxygen	Transient oxygen depletion (TOD)	• Larger oxygen consumption only in hypoxic conditions • Decrease in oxygen consumption with the increase in pulse width	Pratx and Kapp 2020 [80]
Dimensional analysis considering reactive species production, oxygen depletion, and reoxygenation	TOD	• Proportionality of the minimum dose rate for attaining the sparing effect to the product of the diffusion coefficient and concentration of oxygen • Oxygen diffusion distance and drop in intracellular oxygen concentration	Zhou et al. 2020 [81]
Differential equation of oxygen kinetics considering oxygen consumption and reoxygenation	TOD	• Sufficient oxygen depletion only in intermediate oxygen conditions • Reduction of OER with dose rate increments	Petersson et al. 2020 [82]
Physicobiological model considering oxygen metabolism and depletion within spheroids and increased radioresistance of spheroids according to OER and LQ-response	TOD	• Prediction of a hypoxic core in unirradiated tumor spheroids • Engulfment of oxygenated cells by hypoxic core at FLASH • Reproduced hockey-stick survival curve by considering the kinetics of oxygen concentration	Khan et al. 2021 [65]
Reaction-diffusion model considering metabolic and radiolytic oxygen consumption and oxygen supply from microvessels	TOD	• Significance of the sufficient dose and slight hypoxia as well as dose rate for the sparing effect • Prominence of the threshold dose for the FLASH-sparing effect	Rothwell et al. 2021 [83]

Kinetic equation considering the oxygen consumption rate of a tissue and oxygen supply from the capillary as well as radiolytic consumption	TOD	• Enhanced oxygen depletion with dose rate escalation • Saturated oxygen consumption at higher dose rates • Insufficient oxygen depletion for the sparing effect under UHDR	Hu et al. 2022 [84]
Reaction-diffusion equation considering oxygen transport and consumption in vascularized mouse brain tissue model	TOD	• Rapid recovery of oxygen concentration at UHDR • Underestimation of oxygen depletion depending on the vascular density • Requirement of millisecond order-temporal resolution of oximeter	Cui and Prax 2022 [85]
Reaction-diffusion equation for oxygen to model the local oxygen depletion	TOD	• Increased survival of well-perfused normal cells due to the presence of very small, nearly hypoxic regions at FLASH • Increased tumor cell survival at FLASH	Taylor et al. 2022 [86]
Analytical model incorporating OER, oxygen partial pressure, locoregional oxygen diffusion/metabolism, total dose, and dose rate	TOD	• Maximum normal tissue sparing effect in the range of 50-100 Gy/s and marginal sparing effect at > 100 Gy/s • Incapability of interpreting the difference between normal and tumor tissues based on oxygen depletion and OER	Jia et al. 2022 [36]
Model-based analysis of tumor control probability of FLASH-RT with varying dose rates, fractionation schemes, and oxygen concentration	TOD	• Spared tumor growth, but too small to detect in animals • Possibility of substantial loss of tumor control probability in FLASH-RT	Liew et al. 2023 [87]
Analytical model incorporating a time-varying oxygen depletion equation and OER according to LET	TOD	• Sparing effect only at inter-mediate oxygen levels (10–100 mmHg) with < 100 keV/μm LETs in a normal tissue	Song et al. 2023 [88]

Reaction-diffusion equation for reactive species considering cellular scavenging and inter-track interaction	Inter-track interaction	• Well-fitted model to the MC simulation (result for the MC simulation is shown in Table 1) • Requirement of > 90 Gy dose in 0.5 μs for cellular scavenging condition to elicit inter-track interaction of low-LET protons while > 13 Gy in 0.5 μs for water at 4% physoxic condition	Baikalov et al. 2023 [45]
Physicochemical model with ordinary differential equations of reactions of reactive species, biomolecules, and antioxidants	Reactions among reactive species, biomolecules, and antioxidants	• Decrease in ROO$\cdot$ accumulation by dose rate increment • Insufficient oxygen consumption for the sparing effect	Labarbe et al. 2020 [37]
Modeling of dose rate effect on circulating immune cells in a system composed of the heart and numerous organs	Spared circulating immune cells	• Stronger sparing effect on circulating immune cells at FLASH • The requirement of lower dose rate for humans than for mice due to the longer blood circulation time in human • Decrease in the sparing effect with the reduction of dose per fraction	Jin et al. 2020 [89]
Mathematical model that describes the level of surviving lymphocytes in the blood after partial body irradiation for human and mice	Spared circulating immune cells	• Increased surviving lymphocytes with dose rate escalation and decrease in irradiated volume • Maximum sparing of lymphocytes with 10-40 Gy doses within < 1 s exposure time in human • Necessity of a higher dose rate for mice than humans due to the shorter blood circulation time in mice than in human	Cucinotta et al. 2023 [90]

885 **Figure captions**

886 Fig. 1. Breakthroughs in ultra-high dose rate radiation research. The
887 biological effects of ultra-high dose rate (UHDR) have been investigated
888 since the late 1950s, and FLASH-radiotherapy (FLASH-RT) clinical
889 applications are imminent.

Fig. 1.

