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Mechanisms of FLASH effect

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FLASH radiotherapy (FLASH-RT) is a novel radiotherapy technology defined as ultra-high dose rate (≥ 40 Gy/s) radiotherapy. The biological effects of FLASH-RT include two aspects: first, compared with conventional dose rate radiotherapy, FLASH-RT can reduce radiation-induced damage in healthy tissue, and second, FLASH-RT can retain antitumor effectiveness. Current research shows that mechanisms of the biological effects of FLASH-RT are related to oxygen. However, due to the short time of FLASH-RT, evidences related to the mechanisms are indirect, and the exact mechanisms of the biological effects of FLASH-RT are not completely clear and some are even contradictory. This review focuses on the mechanisms of the biological effects of FLASH-RT and proposes future research directions.

KEYWORDS

FLASH radiotherapy, conventional dose-rate radiotherapy, mechanism, oxygen, radiobiology

Introduction

Cancer is one of the leading causes of human death (1). Radiotherapy, as one of the main treatments for cancer, can improve the overall survival time (2) and quality of life (3) and can achieve a radical cure (4) in patients with malignancy. However, the overall anti-tumor effect of radiotherapy is still limited (5). The dose limitation of organs at risk surrounding the cancer leads to the relatively insufficient target dose, and this may be the key factor affecting the anti-tumor effect. Modern radiotherapy technologies, such as volumetric modulated arc therapy, tomotherapy, and proton radiotherapy, can optimize the dose distribution (6, 7) and reduce the toxicity in normal tissues; however, the enhancement of anti-tumor effect is limited (8).

FLASH radiotherapy (FLASH-RT) is a novel radiotherapy technology defined as ultra-high dose rate (≥ 40 Gy/s) radiotherapy. Compared with conventional radiotherapy (CONV-RT), FLASH-RT can effectively reduce the toxicity in normal tissues and provide similar anti-tumor effects, which is defined as the FLASH effect (9). Preclinical studies have confirmed that FLASH-RT can effectively reduce the toxicities in lung (10, 11), intestine (12), brain (13), and skin (14), and retain the anti-tumor effect on cancer (11, 15, 16). Due to these encouraging

results, FLASH-RT is considered as a revolutionary technology in the field of radiotherapy (17). However, due to the short time of FLASH-RT, evidences regarding the mechanism of FLASH effect are indirect. Therefore, the exact mechanisms of the biological effects of FLASH-RT are not completely clear and some are even contradictory.

The purpose of this review is to provide a relatively brief literature review and discuss the mechanism of the FLASH effect and mainly review the research progress in two aspects: physical-chemical mechanism and biological mechanism.

Mechanism of FLASH effect

The mechanism of FLASH effect included the physical-chemical mechanism and biological mechanism, the organs involved include lung, brain, intestine, skin etc (Figure 1 and Table 1).

Physical-chemical mechanism hypothesis

Free radicals produced by radiation are the root cause of radiation damage. Some studies try to explain the effects of

FLASH-RT and COVN-RT by the differences of physical-chemical reaction after radiation.

Oxygen depletion

Oxygen is considered as an important radiation sensitizer (33). Compared with hypoxic tissue, oxygen rich tissue has stronger radiosensitivity under the same radiation conditions (34). As FLASH-RT can complete the irradiation in a very short time (microseconds), the oxygen in the tissue is rapidly exhausted, and it is too fast to be supplemented by the circulating blood. This results in the relative lack of oxygen in the tissue compared with COVN-RT (irradiation completed in a few minutes), which may be one of the reasons why FLASH-RT can protect the normal tissue (35). The basis for the formation of this hypothesis comes from the early *in vitro* studies on bacteria (36) and mammalian cells (37). It is believed that hypoxia can lead to radiation resistance under ultra-high dose rate irradiation, and this resistance effect reached the maximum under nitrogen condition (oxygen concentration: 0%) (37). Hornsey et al. further supported this hypothesis through *in vivo* experiments. When the dose rate was >6 krad/min, the mortality after whole-body irradiation (in the oxygen inhalation

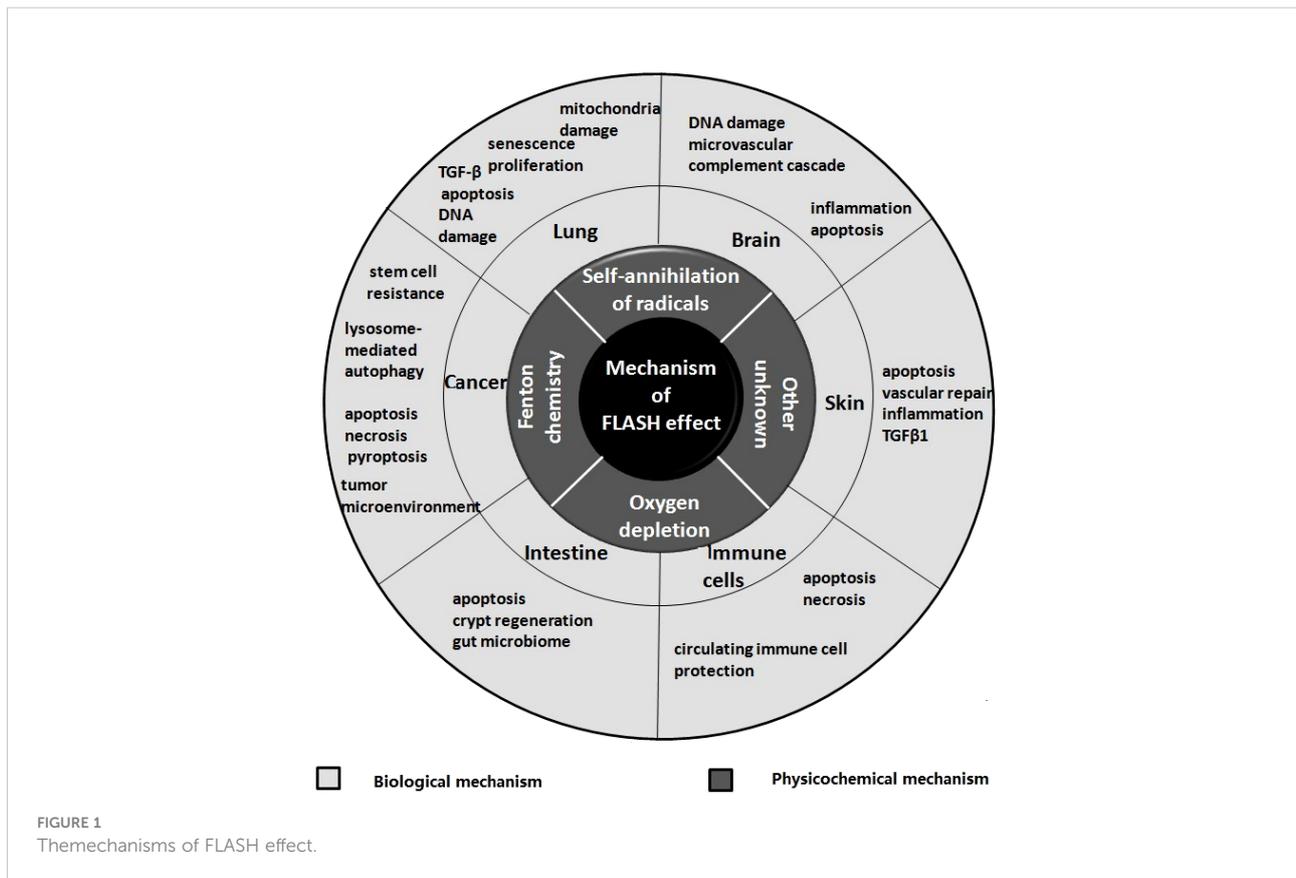


TABLE 1 Summary of published studies on FLASH effect mechanism.

System	Author(s)	Year	Modal	Radiation source	Dose rate (total dose)	Factors relate to Flash effect
Lung	Favaudon V (10)	2014	mice	electron	≥40 Gy/s (17Gy)	TGF-β, Acute apoptosis of vascular endothelial cells
	Fouillade C (18)	2020	mice	electron	≥40 Gy/s (17Gy)	DNA damage, inflammation, proliferation of progenitor, stem cell senescence
	Guo Z (19)	2022	lung fibroblasts	proton	100 Gy/s	mitochondria damage, Drp1-mediated mitochondrial homeostasis
	Buonanno M (20)	2019	lung fibroblasts	proton	1000 Gy/s (>10Gy)	TGFβ, senescence, DNA damage, inflammation
Brain	Montay-Gruel P (21)	2020	mice	electron	5.6×10 ⁶ Gy/s (10Gy)	astrogliosis, complement cascade, inflammation
	Allen BD (22)	2020	mice	electron	2,500 Gy/s (25 Gy) or 5.6×10 ⁶ Gy/s (10Gy)	apoptosis of neurocyte, microvasculature integrity
	Dokic I (23)	2022	mice	proton	120 Gy/s (10Gy)	DNA damage, preservation of microvascular, reduction of microglia/macrophage regulated associated inflammation
Intestine	Levy K (16)	2020	mice	electron	216 Gy/s (14Gy)	a greater number of regenerating crypts due to less DNA damage and apoptosis of crypt base columnar stem cells
	Kim MM (24)	2022	mice	proton	106.2~118.5Gy/s (15,18Gy)	retain the regenerative capacity of crypt cells
	Ruan JL (25)	2022	mice	electron	2.2-5.9 × 10 ⁶ Gy/s (7.5~20 Gy)	spare small intestinal crypts and reduce changes in gut microbiome
	Zhu H (26)	2022	mice	X-ray	>150 Gy/s (10Gy,15Gy)	inflammatory blood cells, pro-inflammatory cytokines and lipid peroxidation
Skin	Velapoulou A (27)	2021	mice	proton	69~124 Gy/s (30Gy, 45Gy)	apoptosis and vascular repair signal pathways, inflammation, TGFβ1
	Bozhenko VK (28)	2019	normal lymphocytes, malignant lymphoma cells	photon	1~4×10 ⁹ Gy/min(1~4Gy)	apoptosis, necrosis
Immune system	Jin JY (29)	2020	computation study	-	0.0017~333 Gy/s (2~50 Gy)	protect circulating blood cells
Breast cancer	Yang G (30)	2021	breast cancer cell	ion beams	10 ⁹ Gy/s (6~9Gy)	radio-resistance of cancer stem cell may associate with the increase of lysosome-mediated autophagy, and the decrease of apoptosis, necrosis and pyroptosis
Ovarian cancer	Eggold JT (31)	2022	mice	electron	210 Gy/s (14Gy)	regulatory T cells and CD8+ T cells infiltration, tumor microenvironment
Glioblastoma	Ohsawa D (32)	2022	mice	electron	66 Gy/s (8Gy×2, 12.5 Gy×2)	anti-tumor immune function

state during irradiation) decreased; however, when the mice were in the nitrogen breathing state during irradiation, the protective effect of ultra-high dose rate radiotherapy was lost (38).

Increasing evidences show that the hypothesis of oxygen depletion cannot fully explain the protective effect of FLASH-RT on normal tissues. Firstly, Jansen et al. measured the change of oxygen content in pure water after FLASH-RT, and found that although FLASH-RT could indeed consume more oxygen than COVN-RT, it could not deplete all oxygen (39). Moreover, when the total dose of FLASH-RT was 10 Gy, oxygen consumption was not detected *in vitro* (39). This contradicted the result that 10

Gy FLASH-RT can better preserve the neural function of mice than COVN-RT in animal experiments (40, 41). Secondly, Epp et al. found that the protective effect of FLASH-RT on mammalian cells occurred when the oxygen concentration is very low (less than 0.5%) (37). Adrian et al. (42) found that FLASH-RT showed a higher cell survival rate than COVN-RT only under hypoxic conditions (oxygen concentration less than 4.4%) and when the total dose exceeded 5-10Gy. One possible explanation is that only when the oxygen concentration is low, FLASH-RT can completely consume the oxygen. However, Gabriel et al. (43) found that the protective effect of FLASH-RT was observed in MDA-MB-231, MCF7 and HeLa cell lines under

normoxic conditions (air oxygen concentration). Moreover, the protective effect of FLASH-RT was also observed in oxygen rich tissues such as lung (10). In addition the oxygen depletion hypothesis cannot explain that FLASH-RT and COVN-RT have similar antitumor effects. Because the tumor tissue is relatively hypoxic, FLASH-RT will lead to tumor cell resistance rather than retain the anti-tumor effect.

Metabolism of peroxidized compounds and Fenton chemistry

Since the oxygen depletion hypothesis cannot fully explain the FLASH effect, Spitz et al. (44) believed that the difference in metabolism of peroxidized compounds and labile iron content between the tumor and normal tissue may be the mechanism of FLASH-RT in reducing normal tissue damage and retaining anti-tumor effect. Compared with tumor tissues, normal tissues retain the metabolic process of peroxidized compounds, therefore, normal tissues have lower peroxidized compounds than tumor tissues, and the labile iron content in normal tissues is lower, which is not conducive to the damage of Fenton chemistry to normal tissues (44). However, Spitz et al. only reasoned from the theoretical model, and subsequent experimental verification, especially the use of animal model of gene knockout of key metabolic enzymes, is very important to verify the Spitz 's theory.

Free radical recombination

Abolfath et al. (45) found that FLASH effect was related to O₂ concentration by simulating DNA damage models under different conditions. In normal tissues (oxygen concentration 4%-5%), oxygen and DNA molecules form reactive oxygen species (ROS) after radiation. Compared with COVN-RT, the concentration of ROS under FLASH-RT was higher, which lead to the reorganization of ROS free radicals. Subsequently, as the ROS load was reduced, the damage of FLASH-RT to normal tissues was lowered. However, in tumor tissues, the presence of hypoxia (oxygen concentration <0.4%), the production of ROS decreased, resulting in the loss of tissue protection. Labarbe et al. (46) further proposed a theoretical model based on the formation and decay dynamics of ROS. It was found that peroxy radicals (ROO) was the key component leading to radiation damage, and there was a correlation between the area under the ROO. curve and the probability of normal tissue complications. Compared with COVN-RT, the production rate of ROO. under FLASH-RT was significantly higher. The reduction of ROO. caused by free radical recombination may play a major role in the tissue protection of FLASH-RT. Lai et al. (47) also concluded by using micro-Monte Carlo simulation method that when the dose rate is

10⁷Gy/s and the dose reached 30Gy, it was difficult to exhaust the tissue oxygen with an initial concentration of 0.01%-21%. Compared with COVN-RT, FLASH-RT has higher instantaneous ROO. concentration, and the decrease of ROO. content caused by self-recombination may be the reason of FLASH effect. However, the theory of free radical recombination only comes from model inference. Because the time of free radical recombination is very short, it is difficult to verify it under experimental conditions. Interestingly, Blain et al. (48) found that the production of H₂O₂ (one kind of ROS) was significantly reduced in FLASH-RT group when compared with that in COVN-RT group though *in vitro* study. Moreover, the decrease degree of H₂O₂ is negatively correlated with the dose rate. When the FLASH dose rate is lower than 100Gy/s, the decrease of H₂O₂ is more dramatic. When the FLASH dose rate exceeded 60000Gy/s, the decrease degree of H₂O₂ reaches a platform (38% ± 4%). However, H₂O₂ cannot represent ROS, future studies are needed to compare the differences of other ROS between FLASH-RT and COVN-RT.

Biological mechanism

Effects of FLASH-RT on stem cells

The reduction of stem cell senescence may play an important role in FLASH-RT protection effect. Unlike apoptosis, senescent cells could secrete pro-inflammatory cytokines, such as IL-6, TGF-β and IL-1α, and lead to subsequent pulmonary fibrosis (18). Moreover, the stem cell senescence hinders the process of cell regeneration after radiation injury (18, 49). Fouillade et al. (49) conducted a preclinical study to evaluate the role of stem cell senescence in the protection of normal tissues by FLASH-RT. C57BL/6J mice were irradiated under FLASH-RT (>40Gy/s, 17Gy) and COVN-RT (<0.003Gy/s, 17 Gy) conditions using electron. They found that compared with COVN-RT group, FLASH-RT had less lung injury and similar anti-tumor effect. Further studies showed that the lung protective effect of FLASH-RT may be related to the retention of stem cell replication ability, because they found that the number of senescence stem cells (reduced or disappeared replication ability) in the FLASH-RT group decreased by 50% compared with the COVN-RT group. More importantly, Fouillade et al. compared the lung injury between TERC-/- mice (mice with extremely short telomeres, simulating the state of stem cells senescence) and wild mice, the phenomenon of FLASH-RT on lung protection disappeared in TERC-/- mice. Yang et al. (30) compared the damage ability of FLASH-RT to tumor stem cells and normal tumor cells *in vitro*. They concluded that under the condition of FLASH-RT (10⁹Gy/s, 6~9Gy), tumor stem cells and normal tumor cells both will undergo apoptosis, scorch, and necrosis after irradiation. However, tumor stem cells have stronger radiation resistance than normal tumor cells. The radiation resistance of cancer stem

cells may be related to the increase of lysosome mediated autophagy. Due to Yang et al. (30) did not compare the damage of FLASH-RT and COVN-RT on tumor stem cells, whether tumor stem cells affect the retention anti-tumor effect of FLASH-RT needs further study.

The retention of the stem cell division ability by FLASH-RT may only partially explain the protective effect of FLASH-RT on normal tissues, and there may be other relevant mechanisms for the retention of the anti-tumor effect (27). Simultaneously, more studies are needed to verify the experimental results of Fouillade et al.

Effect of FLASH-RT on immune function

Radiation damage has been shown to be a sterile inflammatory process (50), and immune function plays an important role in radiation injury (51). TGF- β has been proved to be an inflammatory factor that participates in the process of DNA damage, repair and cellular inflammatory response and promotes the formation of radiation-induced pulmonary fibrosis (52). Several studies show that the expression of TGF- β in FLASH-RT group was significantly decreased when compared with COVN-RT group (10, 20, 27). Fouillade et al (49) studied the role of immune inflammatory change in lung injury after FLASH-RT. The animal model and radiotherapy parameters used in the study were consistent as previously described. The results showed that FLASH-RT had less expression of pro-inflammatory factor gene (EGR1) and lower up-regulation of inflammatory factor (TGF- β 1, NF-KB) than COVN-RT. Zhu et al. (26) reported the changes of immune and inflammatory responses after FLASH-RT(>150Gy/s, 10Gy and 15Gy) irradiation on the intestine of mice(BALB/c). X-ray was used in Zhu's study. They found that compared with COVN-RT, the mice in FLASH-RT group had lower intestinal toxicity, inflammatory blood cells (leukocytes, lymphomas, neutrophils), pro-inflammatory cytokines (TNF- α , IL-6, IL-10) and lipid peroxidation were significantly reduced. Preclinical studies suggest that the increase of chronic neuritis associated with microglia activation may be related to radiation-induced brain injury (53, 54). Montay-Gruel et al. (21) studied the brain damage of mice (C57BL/6J) caused by FLASH-RT(5.6×10^6 Gy/s, 10Gy) and COVN-RT(0.1Gy/s, 10Gy) using electron beam. They found that the expression level of the markers (GFAP, TLR4) that activate astrocyte proliferation in the brain of FLASH-RT group were significantly lower than that in COVN-RT group. Recently, the experimental results of Dokic et al. (23) also supported that FLASH-RT reduced microglia/macrophage regulated inflammation compared with COVN-RT.

Circulating immune cells may have an important impact on the repair of normal tissues after radiotherapy (55) and the anti-tumor effect (56, 57). Therefore, the protection of circulating immune cells by FLASH-RT may be part of the

mechanism of FLASH effect. Jin et al. (29) used computer simulation to evaluate the effects of FLASH-RT and COVN-RT on circulating immune cells. They found that the killing rate of FLASH-RT on circulating immune cells was significantly lower than that of COVN-RT (5%-10% vs 90%-100%). However, it should be noted that this research was only computer simulation, and the research results needed to be verified by experiments. Moreover, only the circulating immune cells were considered in this study, rather than the evaluation of immune cells in immune organs and tumor tissues. Whether FLASH-RT can protect the whole immune function needs to be studied in the future. Eggold et al. (31) evaluated the effect of FLASH-RT on immune cells in tumor by establishing an animal model of peritoneal ovarian cancer. It was found that regulatory T cells decreased and CD8⁺ T cells increased in tumors treated with FLASH-RT(210 Gy/s,14Gy) and COVN-RT(0.126 Gy/s,14Gy). When compared with COVN-RT, FLASH-RT group had significantly more T cell infiltration, especially CD8⁺ T. When radiotherapy was combined with PD-1 inhibitor, the anti-tumor effect of FLASH-RT group was better than that of COVN-RT group. The reliability of the results of Eggold's study needs to be confirmed by more preclinical studies, however, this study suggested that FLASH-RT combined with immunotherapy may have bright prospects.

Immune function may also play an important role in preserving the antitumor effect of FLASH-RT. Recently, Liljedahl et al. (32) used tumor bearing mice to compare the anti-tumor effect of FLASH-RT(66 Gy/s, 8Gy \times 2 fractions and 12.5 Gy \times 2 fractions) and COVN-RT(0.133Gy/s, 8Gy \times 2 fractions and 12.5 Gy \times 2 fractions), and re-challenged the cured mice after radiotherapy to evaluate the long-term anti-tumor effect. They found that FLASH-RT and COVN-RT had similar anti-tumor effect (median survival time: 100 days vs 100 days, $p>0.05$). Cured mice (FLASH-RT: 8 mice; COVN-RT: 6 mice) were then rechallenged with tumor. The results showed that tumor re-growth was not detected in the additional 100 days.

Effect of FLASH-RT on blood vessels

Vascular injury caused by radiotherapy is considered to be an important part of radiation injury (58, 59). Favaudon et al. (10) found that FLASH-RT can reduce the acute apoptosis of bronchial vessels compared with COVN-RT. Two studies focus on brain injury showed that FLASH-RT was superior to COVN-RT in protecting the integrity of microvessels in the brain, which may be conducive to the preservation of cognitive function by FLASH-RT (22, 23). However, the current research evidence only supports that FLASH-RT has less vascular damage than COVN-RT, and the impact of FLASH-RT on the upstream gene regulatory pathway is not clear.

Other possible biological mechanisms

Three preclinical studies show that the protective effect of FLASH-RT on intestinal tract may be related to the protection of intestinal crypt cells by FLASH-RT (16, 24, 25). Ruan et al. (25) also found that FLASH-RT has less impact on intestinal flora than COVN-RT, which may be more conducive to the protection of intestinal function. Guo et al. (19) found that FLASH-RT can reduce mitochondrial damage mediated by Dynamin-1-like protein. Jay et al. (60) believed that FLASH-RT may produce an early transient strong acidic environment, which may be one of the mechanisms of FLASH-RT to protect normal tissues. Ohsawa et al. (61) studied the effect of proton FLASH-RT (40 Gy/s) and COVN-RT (0.05 Gy/s) on DNA damage. They found that compared with COVN-RT, the single strand DNA breakage in FLASH-RT group was significantly reduced, but the double strand DNA breakage was similar. Ohsawa et al. (61) speculated that the FLASH-RT might effectively reduce non lethal damage, such as cell senescence, genomic instability and cell transformation.

Conclusions

The mechanism of FLASH effect include physical-chemical mechanism, biological mechanism and others, which involved oxygen depletion, Fenton effect, free radical recombination, stem cells, immune function, blood vessels, etc. But the published results on the mechanism of FLASH effect can not fully explain the FLASH effect. More studies are needed to clarify the real mechanisms of FLASH-RT and the weight values of different mechanisms.

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Author contributions

BL and DH draft the manuscript, FG, YY, DW, YZ, GF, and TD participated in the data review and collection for the study. XD conceived of the study, and participated in its design and coordination. All authors contributed to the article and approved the submitted version.

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