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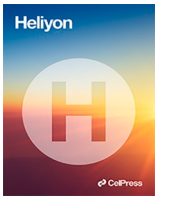

Research article

Effects of ultra-high dose rate radiotherapy with different fractions and dose rate on acute and chronic lung injury in mice

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ABSTRACT

Ultra-high dose rate radiotherapy (FLASH radiation) can naturally render normal tissues around the tumor tissue resistant to radiotherapy. In contrast, the tumor tissue remains sensitive to radiation under the same conditions. However, the effects of different fractions and dose rates on FLASH radiation remain unclear. This study aimed to determine the optimal dose rate and fraction of FLASH radiation for thoracic radiotherapy. Female Balb/c mice aged 6–8 weeks were irradiated with different dose rates (100 Gy/s or 250 Gy/s) and fractions (1, 2, or 4). Survival was observed in mice receiving 30Gy, with lung tissue examined for acute radiation damage 48 h post-radiation. Late radiation pneumonia and survival rates were monitored in mice irradiated with 20 Gy. The median overall survival (OS) was not reached on the 95th day for mice irradiated with 250 Gy/s FLASH radiation, while it was 89.5 days for those irradiated with 100 Gy/s ($P = 0.0436$). Mice irradiated with 30 Gy/2 Fr and 250 Gy/s FLASH had shorter median OS than those with 30 Gy/1F ($P = 0.0132$). However, there was no significant difference in OS between mice irradiated with 30 Gy/2 F and 30 Gy/4 F. Survival curves for mice receiving 20 Gy showed no significant difference in toxicity between different dose rates and fractions. FLASH radiation at 250 Gy/s reduced the incidence of acute radiation pneumonitis in mice compared to 100 Gy/s. Different fractions of irradiation influenced survival in mice, but they were only observed in acute radiation reactions and not chronic radiation reactions. Among the tested fraction methods, fraction 2 had the worst impact on the survival of mice, while fractions 1 and 4 showed similar effects and improved survival compared to fraction 2.

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1. Introduction

Tumors pose a significant threat to human life and health, now ranking as the leading cause of death [1]. Radiotherapy is vital in cancer treatment [2]. Palliative radiotherapy offers relief from symptoms and improves the quality of life of patients [3], while radical radiotherapy can extend survival and potentially cure [4]. However, current radiotherapy techniques limit the therapeutic dose delivered to the tumor due to the inevitable damage to normal tissues, thereby imposing a maximum dose constraint on the organs at risk [5,6]. A key challenge in advancing radiotherapy technology is exploring methods to increase the radiation dose to the tumor target without increasing the radiation dose to nearby normal tissues. In radiation oncology, oncologists primarily achieve this by reducing the dose to normal tissues around the tumor to an acceptable dose by improving the conformal degree of the radiotherapy target and leveraging the proton ray Bragg peak [7–10].

Since 2014, some researchers have explored using ultra-high dose-rate radiation for radiotherapy, which is hundreds of times higher than the dose rate of conventional radiotherapy. The results showed that normal tissues exhibit radiation resistance under ultra-high dose-rate irradiation, while tumors remain sensitive [11–15]. This breakthrough offers a way to address the limitations of traditional tumor radiotherapy. Ultra-high dose-rate radiotherapy can naturally render normal tissues surrounding the tumor resistant to radiotherapy while maintaining the sensitivity of tumor tissue to radiation under the same conditions [11,12,14,16]. As a result, ultra-high dose-rate radiotherapy has rapidly become a research hotspot in radiotherapy.

Numerous studies have explored the protective effects of ultra-high dose-rate radiotherapy on various tissues, including the brain [17], lung [14,16], and small intestine [15,18,19] in mice. Additionally, research has investigated its efficacy in tumor control in tumor-bearing mice [16], its application in radiotherapy of skin squamous cell carcinoma in cats, and skin protection in mini-pigs [20]. Despite variations in radiation parameters, timing, and dosage across these studies, they consistently arrived at the same conclusion.

In studies investigating the mechanism of ultra-high dose-rate radiotherapy, researchers have highlighted the role of reduced oxygen consumption as a critical factor in protecting normal tissues [21–23]. Consequently, a super-rapid implantation dose is a prerequisite for ultra-high dose-rate radiotherapy [24–26]. However, the specific conditions necessary for super-rapid radiotherapy remain uncertain. Questions arise about the duration of irradiation required for rapid radiotherapy. Electrons are more adept at achieving short implantation of ultrahigh dose rates than other rays. However, achieving the same pulsed dose rate with photons presents more challenges. Ultra-high dose-rate photons have been reported previously; however, photons typically operate under an average dose condition (>40 Gy/s) of ultra-high dose-rate radiotherapy with longer pulse time and shorter pulse dose rate. Although the pulse structures of photons and electrons are different, research confirms that both types of radiation can induce the FLASH effect, effectively killing tumor tissue while protecting normal tissue when the average dose rate exceeds 40 Gy/s [14,16]. Therefore, leveraging the resources available in our lab, we propose that varying dose rates and fractions could produce the same FLASH effect. Finding the minimum dose rate to trigger the FLASH effect or determining if multifraction can also induce the FLASH effect significantly alleviates the challenges in the research and development of future ultra-high dose rate radiotherapy equipment. This would increase the likelihood of ultra-high dose rate radiotherapy becoming a new mode of radiotherapy.

To investigate these possibilities, we designed experiments in mice using different dose rates and fractions to assess whether the FLASH effect could be elicited under the new experimental conditions.

2. Materials and methods

2.1. Irradiation devices

Ultrahigh dose-rate FLASH irradiation was performed using the PARTER platform at CTFEL, Chengdu, China. This platform utilizes a superconducting linac capable of generating 6–8 MeV electrons with an adjustable mean current of up to 10 mA and an energy spread of less than 0.2 % (root mean square measured at a beam energy of 8.2 MeV).

2.2. Dosimetry

The experimental setup and team members were identical to those detailed in a previous paper published in 2022. Dosimetry information for this study is available in the article published in 2022 [16]. In our experiment, we employed two different dose rates: 100 Gy/s and 250 Gy/s, respectively. The total irradiation dose was either 30 Gy or 20 Gy, with irradiation times of 0.3s, 0.12s, 0.2s, and 0.08s for the single fraction experiment. In the multifraction experiment, the interval between each radiation session was 8 min.

2.3. Animal experiment and ethics statement

Ultra-high dose rate X-rays with dose rates of 100 Gy/s and 250 Gy/s were used to irradiate the thorax of Balb/c female mice aged 6–8 weeks; the irradiation field was a 2 × 2 cm square field, the upper boundary was the line of the lower edge of the auricle of mice, the lower boundary was 2 cm down the upper limit. The left and right boundaries were 1 cm apart from the median line of mice. Vertical irradiation from the backs of the mice was employed, delivering irradiation doses of 30 Gy and 20 Gy.

We carried out two different experiments. The first was irradiation at a dose rate of 250 Gy/s with different fractions under the condition of the same total irradiation dose, which was called a different fraction experiment. The second was a single irradiation at dose rates of 250 Gy/s and 100 Gy/s under the condition of the same total dose, which is called a different-dose rate experiment.

(Experimental groups are provided in Table 1).

After irradiation, mouse survival and weight were monitored weekly. Additionally, the mice were irradiated with ultra-high dose-rate X-rays using different fraction modes of 250 Gy/s, including 30 Gy/1F, 30 Gy/2F, and 30 Gy/4F. The total duration of radiation exposure in all three groups was 0.12s, precisely controlled by CTFEL. Mice receiving one or three intervals of irradiation remained in the lab during the process, which lasted about a couple of seconds. Five mice from each 30 Gy irradiated group were euthanized 48 h post-irradiation (pi) for hematoxylin and eosin (HE) and Masson's trichrome staining of lung tissue. In different fraction and dose rate experiments, mice received 20 Gy irradiation. The process was identical to the one described above, including the irradiation field and methods. However, lung tissue samples were not collected from mice irradiated with 20 Gy 48 h pi but were observed for overall survival (OS).

Before the experiments, animal ethics approval was obtained from the Animal Ethics Committee of Mianyang Hospital (approval number: P2020032). The experiments were conducted following the guidelines for the assessment of human endpoints in animal experiments published in China in 2018.

2.4. Histopathology process

We utilized a microscope to examine the normal tissue sections stained with HE at 200 × or 400× magnification. Mice irradiated with 30 Gy were observed for 95 days, With their survival tracked daily. Fresh tissue was fixed for at least 24 h. The sections were flattened and placed in a dehydration box. After dehydration, the waxed tissue was embedded in an embedding machine and labeled accordingly. Slices measuring 3 μm were prepared, and the wax was removed before staining with hematoxylin and eosin.

2.5. Statistical analysis

Survival curves were compared using the Kaplan–Meier method with a log-rank test. All statistical tests were conducted at a significance level of 5 %. A two-tailed p-value **<** 0.05 indicated a statistically significant difference, and 95 % confidence intervals (CIs) were also calculated.

3. Results

3.1. Survival differences of mice irradiated by different dose rates of 30 Gy irradiation

Under ultra-high dose rate X-ray irradiation of 250 Gy/s and 100 Gy/s, we conducted experiments involving single-fraction, two-fraction, and four-fraction irradiation on the thorax of mice. The total irradiation doses were 30 Gy and 20 Gy, respectively. Mice irradiated with 30 Gy were observed for acute radiation pneumonia, while those irradiated with 20 Gy were observed for chronic radiation reactions. In the experiments comparing the effects of different dose rates, the median overall survival (mOS) of mice irradiated with 250 Gy/s FLASH radiation was not reached on the 95th day post-exposure. In contrast, mice irradiated with 100 Gy/s FLASH radiation have a mOS of 89.5 days ($P = 0.0436$) (Fig. 1a).

3.2. Morphological changes under the microscope

Masson's trichrome staining of the lung sections revealed relatively normal alveolar structures. In the 100 Gy/s and 250 Gy/s groups, there was increased staining of collagenous fiber (blue staining area) around the alveoli compared to the control group. However, upon microscopic examination, the blue-stained area of the 250 Gy/s group was light and uniform, whereas in the 100 Gy group, it seemed more disorganized (Fig. 1b).

3.3. Survival differences in mice irradiated by different fractions

The mOS of mice irradiated with 30 Gy/2F and 250 Gy/s ultra-high dose-rate radiation was 70 days, shorter than those irradiated with 30 Gy/1F ($P = 0.0132$). Similarly, at the same dose rate, the mOS of mice irradiated with 30 Gy/2F was shorter than those irradiated with 30 Gy/4F (undefined mOS), but the difference was not statistically significant (Fig. 2a 2b).

Regarding morphological changes in lung tissue under the same dose rate and different fraction conditions, the most severe damage was observed in the two-fraction irradiation group. In contrast, the mildest damage occurred in the single fraction group. The damage in the four-fraction groups was intermediate. Morphological changes included incomplete alveolar structure, infiltration of inflammatory cells, necrosis and shedding of bronchial mucosal epithelial cells, and hypertrophy of the vascular walls.(see. Fig. 2c)

Table 1
Experimental group details.

	250 Gy/s			100 Gy/s
Fractions	1F	2F	4F	1F
Total dose	30Gy	30Gy	30Gy	30Gy
Total dose	20Gy	20Gy	20Gy	20Gy

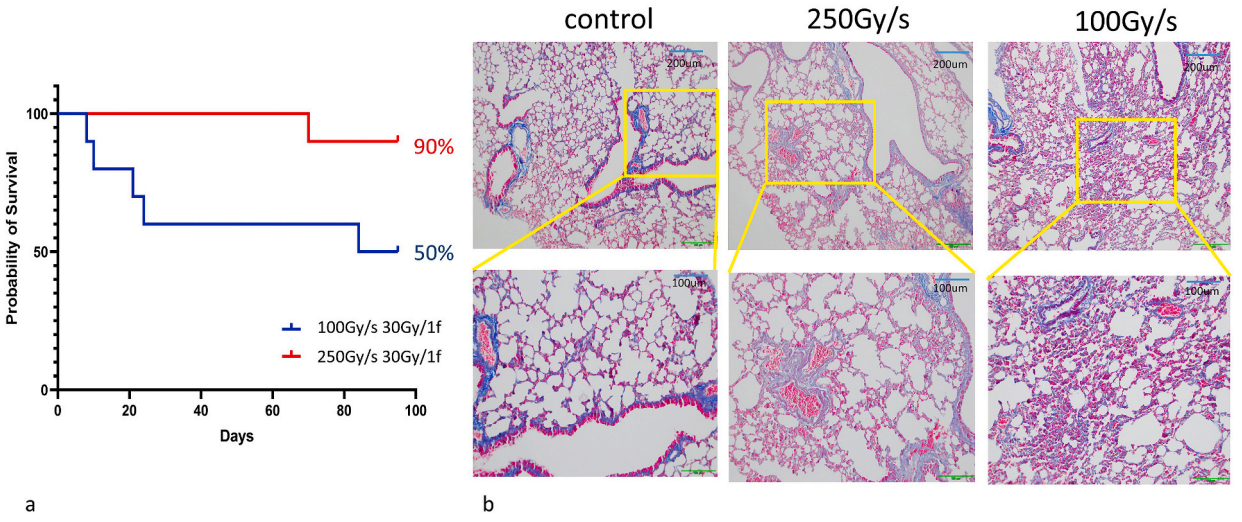

Fig. 1. (a)Mice irradiated by 250 Gy/s X-ray exhibited better survival than mice irradiated by 100 Gy/s. (b) Masson's special staining of the lung sections showed that alveolar structures were relatively normal, and collagenous fiber staining (blue-staining area) around the alveoli was more obvious in the 100 Gy/s and 250 Gy/s group than in the control group. The blue-staining area of the 250 Gy/s group is light and uniform, and the blue-staining area of the 100Gy group seems to be more disorderly. In different dose rate experiments, higher dose rate chest irradiation causes less tissue damage than lower dose rate irradiation.

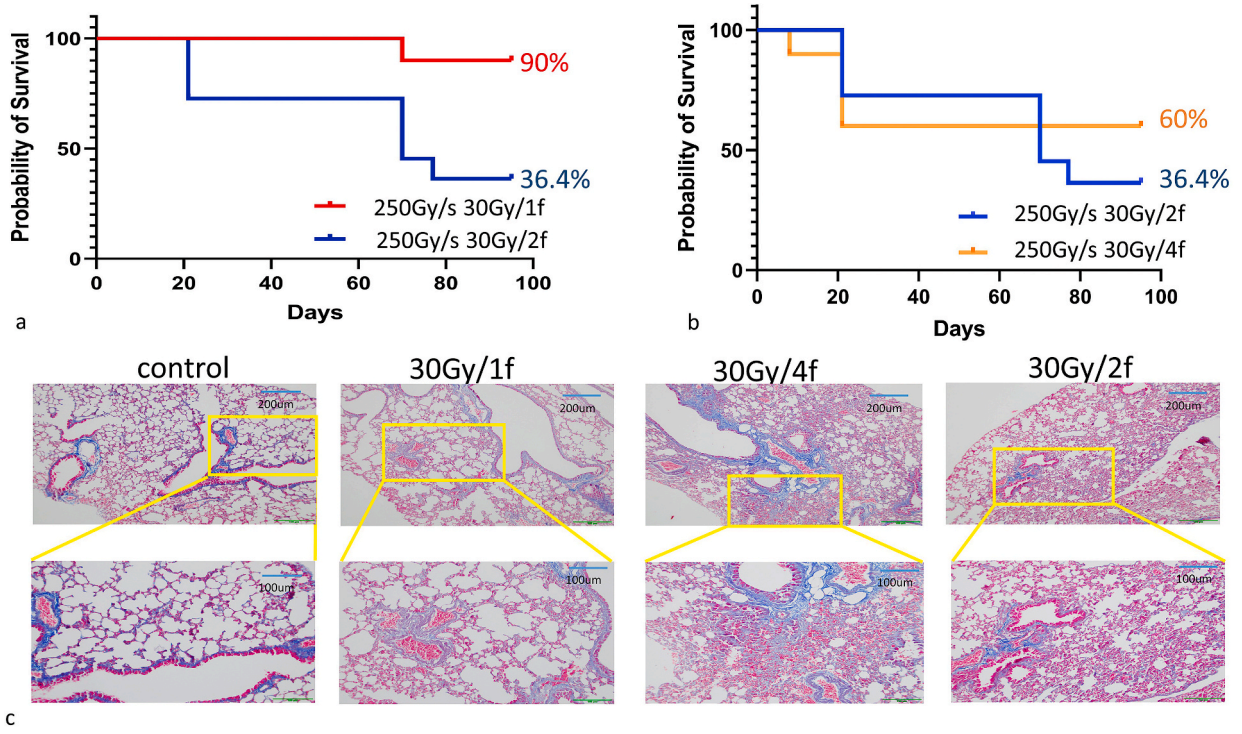

Fig. 2. (a)Mice received 250 Gy/s dose rate in different fractions experiments. Median survival rate of mice exposed to 1 fraction irradiation was higher than that of mice exposed to 2 fractions and 4 fractions. The median survival rate of mice exposed to 1 fraction and 4 fractions showed statistical differences. (b) Median survival rate of mice exposed to 2 and 4 fractions differed but had no statistical significance. (c) Masson's special staining of the lung sections showed that the single fraction group was the least damaged.

3.4. No significant differences in mice survival irradiated with 20Gy

The mOS of mice irradiated with 20 Gy was comparable across the various groups, in experiments involving different fraction or dose rates. Specifically, the mOS was 132days in 250 Gy/s 20 Gy/2F; 142days in 100 Gy/s 20 Gy/1F; 125days in 250 Gy/s 20 Gy/2F and 135days in 250 Gy/s 20 Gy/1F. There were no significant differences between the groups. (Fig. 3).

4. Discussion

Ultra-high dose-rate FLASH radiotherapy (FLASH-RT) is a new radiotherapy modality. This approach selectively eliminates tumor cells while sparing surrounding normal tissues, a phenomenon known as the FLASH effect [14,26]. The FLASH-RT has shown promising prospects in animal experiments [12–17,19,27], suggesting it could address a critical challenge in radiotherapy development by enhancing tumor target dose delivery while reducing the radiosensitivity of adjacent normal tissues. However, there is no unified conclusion regarding the trigger conditions for FLASH-RT [19,28]. Although an average dose rate of 40 Gy/s [14,22,29] was considered a sufficient condition for FLASH-RT, divergent experimental outcomes persist, with some studies reporting an average dose rate exceeding 40 Gy/s to trigger the FLASH effect and others reporting failure to induce the FLASH effect [28,30,31].

Electron [14,22,32,33], photon [32,34], and proton [27,35,36] rays have been successfully used in FLASH-RT experiments. However, whether each ray possesses unique parameters that trigger the FLASH effect is uncertain. These distinct triggering conditions could hold crucial insights into the underlying mechanism of the FLASH effect. While most studies showed that an average dose rate of over 40 Gy/s is pivotal for initiating FLASH-RT, the average dose rate is influenced by two elements [37]: the total dose and the duration time of the rays. Thus, various combinations of doses and durations may achieve the same average dose rate. For instance, the dose is doubled when the irradiation time is doubled, so the average dose rate is constant. Alternatively, halving the irradiation time doubles the average dose rate. Whether these different doses and irradiation times combinations yield equivalent FLASH effects at the same average dose rate remains uncertain.

In this study, a specific pulse dose rate was used. The source-skin distance can be used to adjust the average dose rate to achieve different effects. The total dose was adjusted based on the duration of radiation exposure. We observed varying effects on pulmonary fibrosis in mouse lung tissue with different average dose rates. Notably, differences in survival rates were first noted in acute radiation lung injury cases.

The effect of different fractions on FLASH-RT was discovered in 1967, where the FLASH effect was observed in one fraction but not in the other two, as observed in HeLa cells [19,38]. The same result was observed in the crypts of the small intestine of mice. In our study, The mOS of mice receiving the prescribed dose of 30 Gy/1F was better than those receiving 30 Gy/2F and 30 Gy/4F. Furthermore, a dose rate of 250 Gy/s demonstrated a more protective effect on normal tissue than 100 Gy/s. Although the exact explanation for this phenomenon remains unclear, we speculate that oxygen consumption may play a role [24,25,39].

Oxygen consumption is the mainstream theory explaining the FLASH effect [40]. Compared to conventional radiotherapy, FLASH-RT has a very high dose rate, potentially leading to rapid oxygen depletion in normal tissues. This phenomenon induces relative hypoxia in normal tissues, resulting in relative radiation resistance. The degree of oxygen consumption by FLASH-RT is related to the total dose and average dose rate [41]. The thorax radiation of 30Gy/1F and 30Gy/2F were to examine acute radiation lung injury in mice. The radiation of 30Gy/2F is actually two cycles of 15Gy/1F with the interval of 8 min.The total dose of one shot was only 15Gy in 30Gy/2F radiation mode, and the total oxygen consumption was less than the total oxygen consumption of 30Gy/1F. in the 30Gy/2F radiation mode, the cells rapidly reoxygenated, which increased the sensitivity of lung tissue to radiotherapy [42–44].

This study has certain limitations. We did not include a group subjected to conventional dose rate irradiation. As a result, we could not directly compare the degree of pathologic change in normal tissues in the conventional dose rate radiotherapy group and the ultra-high dose rate X-rays of 100 Gy/s under the same dose conditions. However, it is possible to indirectly judge whether 100 Gy/s ultra-high dose rate irradiation has a normal tissue protection effect by comparing it with published data on 30 Gy thorax irradiation at the

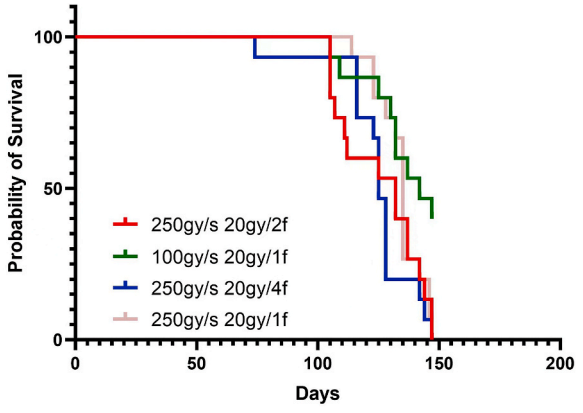

Fig. 3. In the experimental mice irradiated 20 Gy, no positive results were observed in the different fractions or dose rate experiments.

conventional dose rate in mice. This comparison allows us to infer whether the 100 Gy/s irradiation group triggered the FLASH effect. Setting up a conventional dose-rate irradiation group would enhance the completeness of the results.

5. Conclusions

Our study revealed that irradiation with different fractions influenced mouse survival, particularly in acute and not chronic radiation reactions. Notably, among the three different fraction methods examined in our experiment, fraction 2 had the most detrimental effect on mouse survival. In contrast, fractions 1 and 4 had a similar impact on survival, and the survival time surpassed that of fraction 2. This study's findings may inform the development of novel therapeutic approaches aimed at enhancing the efficacy of FLASH-RT while minimizing normal tissue toxicity. Future studies should explore whether different types of radiation, such as electron, photon, and proton rays, possess unique parameters that trigger the FLASH effect, providing insights into the underlying mechanisms of this phenomenon.

CRediT authorship contribution statement

Feng Gao: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. **Binwei Lin:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration. **Yiwei Yang:** Data curation, Investigation, Methodology. **Dexin Xiao:** Methodology. **Zheng Zhou:** Methodology. **Yu Zhang:** Investigation. **Gang Feng:** Methodology. **Jie Li:** Methodology. **Dai Wu:** Conceptualization. **Xiaobo Du:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Qiuling Shi:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Consent for publication

Not applicable.

Data availability statement

Not applicable.

Animal experiments

Ethical approval was obtained from the Animal Ethics Committee of Mianyang Hospital (approval number: P2020032).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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