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Current methodology in FLASH radiotherapy research

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Abstract

FLASH radiotherapy (FLASH-RT) is a novel irradiation technique that delivers radiation at ultra-high dose rates and has demonstrated the potential to reduce radiation-induced damage to normal tissues while preserving anti-tumour efficacy. Although the mechanisms responsible for the FLASH effect remain under investigation, numerous preclinical studies have reported promising results.

This review presents the main methodologies used to investigate the biological effects of FLASH-RT in both in vitro and in vivo studies. This review provides an overview of the principal in vitro and in vivo methodologies used to investigate the biological effects of FLASH-RT, highlighting their applications in evaluating treatment response and exploring the mechanisms underlying the FLASH effect. The applications and significance of these methods in evaluating treatment efficacy, DNA damage, and normal tissue response are highlighted

Further standardisation of experimental protocols will be essential to improve the reproducibility of FLASH studies and facilitate the future clinical implementation of this promising radiotherapy technique.

Keywords: FLASH, radiotherapy, animal models, in vitro assays, in vivo assays

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Introduction

Cancer continues to be one of the leading causes of death globally and represents a major public health challenge. Although significant progress has been made in cancer diagnosis and treatment, many malignancies are still associated with limited therapeutic options and high mortality rates. In 2022, approximately 20 million new cancer cases were diagnosed worldwide. Lung cancer was the most frequently diagnosed cancer (12.4% of all new cases), followed by female breast cancer (11.6%) and colorectal cancer (9.6%) [1]. The global number of cancer cases is expected to increase to approximately 35 million by 2050, emphasising the urgent need for effective prevention strategies focused on reducing risk factors such as tobacco use, obesity, and infections [1].

Radiotherapy (RT) is a cornerstone of cancer treatment and is used in the management of a wide range of malignancies, including brain, lung, breast, head and neck, prostate, and colorectal cancers. However, tumours located close to critical organs or characterised by radioresistance, hypoxia, or extensive local invasion remain particularly challenging to treat, as delivering a curative radiation dose is often limited by the risk of severe damage to surrounding healthy tissues [2, 3]. Although chemotherapy is frequently combined with RT to improve treatment outcomes, its use is often restricted by systemic toxicity, the development of drug resistance, and limited efficacy in certain tumour types. Therefore, there is a growing need for novel RT strategies that enhance treatment effectiveness while reducing damage to normal tissues.

Conventional RT versus FLASH-RT

Conventional RT (CONV-RT) has long been a basis of cancer treatment and remains the standard of care for many malignancies. Recently, FLASH-RT, which delivers radiation at ultra-high dose rates, has emerged as a promising approach with the potential to improve the therapeutic ratio.

FLASH-RT has been investigated primarily using electron and proton beams, with preclinical studies demonstrating comparable normal-tissue sparing and anti-tumour responses across these modalities, suggesting that the FLASH effect may be observed with different beam types and dose rates, whereas CONV-RT most commonly relies on photon beams delivered at conventional dose rates [4].

The fundamental difference between CONV-RT and FLASH-RT lies in the radiation dose rate. CONV-RT is typically delivered at dose rates of approximately 0.01–0.1 Gy/s, whereas FLASH-RT uses ultra-high dose rates exceeding 40 Gy/s, enabling the full prescribed dose to be delivered within milliseconds. This substantially greater dose rate is considered the key factor responsible for the unique biological effects observed with FLASH-RT [5]. The extremely short irradiation time in FLASH-RT suggests an early modulation of radiochemical processes that are dependent on oxygen levels within the irradiated volume [6]. FLASH-RT may rapidly deplete local oxygen, leading to transient radiation-induced hypoxia [6, 7], which selectively protects normal tissues from radiation-induced damage [6, 34]. This oxygen-dependent mechanism is considered central to the so-called FLASH effect. In contrast, such a rapid radiochemical oxygen depletion does not occur during CONV-RT due to its much lower dose rate, which results in continuous oxygen replenishment throughout the irradiation process.

Beyond oxygen depletion, several non-mutually exclusive hypotheses have been proposed to explain the FLASH effect. These include dose-rate-dependent changes in reactive oxygen species chemistry and radical–radical recombination, which may reduce oxidative damage in normal tissues while preserving the cytotoxic effects of irradiation in tumours [8]. Differences in DNA damage induction and repair have also been suggested, particularly with respect to the formation of complex or clustered DNA lesions and the capacity of normal and tumour cells to activate DNA damage response pathways [9]. In addition, FLASH-RT may influence mitochondrial function, oxidative metabolism, and the balance between pro-survival

and cell-death signaling [10]. At the tissue level, modulation of immune and inflammatory responses, preservation of circulating immune cells, and alterations in vascular or stromal responses have been proposed as potential contributors to normal-tissue sparing [11].

FLASH-RT therefore represents a promising advancement in radiation oncology, offering a potentially improved therapeutic ratio through reduced normal tissue toxicity while maintaining tumour control.

However, clinical evidence remains limited, and most of the current knowledge is derived from preclinical studies. Therefore, comprehensive in vitro and in vivo investigations are essential to better understand the biological mechanisms underlying the FLASH effect and to support its safe translation into clinical practice.

In vitro studies of FLASH-RT

In vitro studies play a fundamental role in FLASH radiobiology by providing controlled experimental conditions for investigating the cellular and molecular responses to ultra-high dose rate irradiation. These models enable the evaluation of key biological endpoints, including cell survival, DNA damage, and oxygen-dependent mechanisms, and serve as an essential first step in understanding the biological basis of the FLASH effect.

Clonogenic test

Clonogenic assays are a standard in vitro method in radiobiology used to evaluate the long-term survival and reproductive capacity of cells after irradiation, effectively reflecting all major forms of radiation-induced cell death. The assay can be performed using two main approaches: pre-plating or post-plating [12]. In the pre-plating method, cells are first seeded at appropriate densities, allowed to attach, and then irradiated directly in culture vessels. In the post-plating method, cells are irradiated first, subsequently harvested, counted, and re-seeded at defined densities to assess their colony-forming ability after incubation [12].

The clonogenic assay is still considered the standard method for measuring long-term cell survival after irradiation and remains an important tool for confirming results in FLASH-RT research. With strict control of experimental conditions, such as time outside the incubator, culture medium volume, cell density, and well-matched beam and dose parameters in CONV-RT and FLASH-RT groups, the method can provide reliable and reproducible results and allow the analysis of cellular responses under different oxygen conditions [13].

Comet assay

The comet assay is a widely used technique for assessing DNA damage and repair at the single-cell level following exposure to ionising radiation. In this technique, cells are immobilised within a thin layer of low-melting agarose on microscope slides and subsequently subjected to lysis in a detergent- and salt-rich solution that removes membranes and nuclear proteins, leaving DNA organised as nucleoids [14]. Exposure to alkaline conditions causes DNA strands to unwind and acquire a negative charge, enabling fragmented DNA to migrate through an electrophoretic field towards the anode [14]. After fluorescent staining, the resulting structures resemble comets under the microscope, with a distinct head and tail, where the extent of DNA migration corresponds to the degree of strand

breakage [15]. The comet assay allows for direct comparison of DNA damage induced by FLASH-RT and CONV-RT under varying experimental conditions. It can also be applied to assess how both dose rate and total delivered dose influence the ex vivo radioprotective effect observed between FLASH- and CONV- irradiation on blood cells in conditions of low oxygen availability [13].

Oxygen concentration measurement

Since the FLASH effect is strongly influenced by oxygen availability, accurate measurement and control of oxygen concentration are essential in FLASH-RT research. Monitoring oxygen levels helps to better understand the transient oxygen depletion believed to contribute to the radioprotective effect observed in normal tissues during ultra-high dose rate irradiation.

Oxygen concentration is commonly measured using oxygen sensitive fluorescent or phosphorescent probes [16]. These include fiber optic probes coated with oxygen sensitive fluorescent compounds and water-soluble molecular probes such as Oxyphor 2P (**O**xyphor LLC, Havertown, PA, USA), which enable real time monitoring of local oxygen levels during irradiation [17, 18].

In vivo studies of FLASH-RT

In vivo studies play a crucial role in the preclinical evaluation of FLASH-RT, as they allow the assessment of its biological effects in complex living systems that better reflect clinical conditions.

Animal models

Animal models are essential for investigating the biological effects, therapeutic efficacy, and normal tissue toxicity of FLASH-RT under physiological conditions. They provide a platform for evaluating both tumour response and radiation-induced damage in healthy tissues, enabling direct comparison between

FLASH- and CONV-dose rate irradiation.

Mice are the most commonly used animal model in FLASH-RT research because of their well-characterised genetics, wide availability of tumour models, and suitability for investigating both tumour response and normal tissue toxicity.

Rats are also frequently used, particularly in studies focusing on normal tissue responses and radiation-induced injury, owing to their larger size and ease of physiological assessment. Larger animal models, such as mini pigs, provide greater translational value because their skin and tissue characteristics closely resemble those of humans, making them particularly useful for evaluating radiation toxicity and tissue sparing.

Total body irradiation

Total body irradiation (TBI) can be used in preclinical FLASH studies to irradiate the whole organism under controlled and reproducible conditions, enabling comparison between FLASH- and CONV- dose rate exposure. It allows assessment of systemic effects, including hematopoietic and immune responses as well as normal tissue toxicity and tumour control.

The relatively uniform dose distribution makes it a useful model for studying dose rate-dependent biological differences. TBI involves exposing the entire organism to ionising radiation and serves as both a preclinical research model and a component of conditioning protocols prior to hematopoietic stem cell transplantation. The technique is designed to eliminate residual malignant cells while inducing sufficient immunosuppression to support successful donor cell engraftment [19].

In preclinical FLASH studies, TBI is most commonly performed in mouse models, including immunocompetent strains and immunodeficient mice bearing haematological malignancies [20]. During irradiation, animals are positioned so that the entire body is encompassed within a large radiation field, allowing delivery of a homogeneous dose. Large radiation fields and an extended source-to-skin distance (SSD) are commonly used to achieve dose uniformity, while individualised treatment planning may incorporate compensating filters, shielding of radiosensitive organs, and in vivo dosimetric verification [19, 20].

Tumour growth delay

Tumour growth delay (TGD) is an in vivo measure used to determine how a treatment influences the rate of tumour expansion by tracking how long it takes for a tumour to reach a defined size compared with untreated controls. It is widely used in radiobiology because it provides a functional readout of treatment efficacy over time rather than a single endpoint. In FLASH research, TGD is valuable as it enables direct comparison between FLASH and CONV- irradiation in terms of tumour growth kinetics under the same experimental conditions, helping to assess whether dose rate affects tumour control.

The assay is most commonly performed in mouse models bearing subcutaneous or orthotopic tumours [21]. After tumour implantation and growth to a predefined volume, animals are treated with either FLASH- or CONV- irradiation [21]. Tumour dimensions are then recorded at regular intervals using calipers or imaging techniques such as MRI [22, 23], and the measurements are converted into tumour volume estimates. To minimise the influence of differences in baseline tumour size, volumes are frequently normalised to pre-treatment values [22]. The resulting growth curves allow comparison of tumour progression and treatment response between the experimental groups.

Assessment of normal tissue toxicity

RT aims to maximise tumour control while minimising damage to surrounding normal tissues. Therefore, the evaluation of normal tissue toxicity is a critical component of preclinical studies investigating novel irradiation techniques, dose delivery approaches, or radioprotective strategies. In vivo models provide an opportunity to characterise tissue responses under controlled experimental conditions and to establish dose–response relationships that can be used to compare treatment modalities.

Normal tissue toxicity is evaluated in vivo by irradiating selected healthy tissues in animal models under controlled experimental conditions. After irradiation, animals, mainly mice, are monitored over time, and radiation-induced tissue damage is assessed using standardised toxicity scoring systems based on observed clinical changes [25]. The obtained toxicity scores are used to establish dose–response relationships and compare tissue sensitivity between different irradiation conditions [25]. Depending on the experimental

model and the investigated tissue, toxicity assessment may include standardised scoring systems or general clinical monitoring of radiation-induced changes in healthy tissues, such as skin reactions and other treatment-related effects [26].

γH2AX immunofluorescence assay in in vitro and in vivo models

The γH2AX immunofluorescence assay is widely used in FLASH radiobiology to assess radiation-induced DNA damage in both in vitro and in vivo experimental models. It enables sensitive detection of early DNA damage induced by ionising radiation and allows direct comparison between FLASH and CONV- dose rate irradiation. In vitro, the assay is commonly performed using irradiated cultured cells, whereas in vivo applications involve the analysis of tissue samples collected after irradiation. This technique is particularly important for evaluating whether ultra-high dose rates reduce radiation-induced genotoxic stress in normal tissues.

In this method, biological samples are harvested at defined time points after irradiation and fixed prior to immunolabelling with an antibody against phosphorylated histone H2AX (γH2AX), which serves as a marker of DNA double-strand breaks, together with a nuclear counterstain such as DAPI [24]. The specimens are then examined using confocal microscopy, and the resulting γH2AX fluorescence is quantified to estimate the level of radiation-induced DNA damage [24]. The resulting data can be used to characterise the distribution and extent of DNA damage within the analysed samples.

Challenges in FLASH-RT quality control

Despite promising biological results, FLASH-RT faces some technical difficulties which have to be resolved before wider employment in clinical trials. Accurate dosimetry is a crucial part of RT delivery, and the methods used for CONV- irradiation are not as robust when used with FLASH. A large number of facilities using FLASH are not able to reach a 5% accuracy of dose delivery [27]. The ionisation chambers which are standard in CONV-RT show significant inaccuracies when used for FLASH dosimetry due to ion recombination [28]. Using correction factors or more accurate ultra-thin chambers might be the solution improving measurement accuracy [29].

Other methods, such as alanine dosimetry and graphite calorimeters have been used to some success for FLASH dosimetry [30]. Establishing passive dosimetry methods such as alanine and radiochromic films is crucial, especially for preclinical studies, and methods for real-time measurements need to be stablished for effective clinical use [31]. Due to the ultra-short irradiation time, new solutions such as detector arrays are being constructed for beam profile characterisation and quantification of the pulse-to-pulse stability [32]. It is also important to note, that FLASH systems might experience high dose rate fluctuations, which is why quality assuranceprograms need to be established to assess their performance [33].

Conclusions

FLASH-RT has emerged as a promising approach with the potential to improve the therapeutic ratio by reducing normal tissue toxicity while maintaining anti-tumour efficacy. The reliable evaluation of the FLASH effect requires the use of complementary in vitro and in vivo experimental models, as no single method is sufficient to fully characterise its biological response. In vitro assays, including clonogenic assays, comet assays, and oxygen measurements, provide valuable insights into cell survival, DNA damage, and the proposed mechanisms underlying the FLASH effect. Animal models further enable the assessment of tumour control, systemic responses, and normal tissue toxicity under physiologically relevant conditions. Tumor growth delay (TGD), γH2AX analysis, immunofluorescence assays, and normal tissue toxicity assessments are among the methods used to evaluate treatment efficacy and radiation-induced biological effects. TGD is primarily used in in vivo tumor models, whereas γH2AX analysis and immunofluorescence can be applied to both in vitro and in vivo systems. Total body irradiation (TBI) represents a distinct irradiation approach used in specific experimental settings, particularly in vivo studies.

Despite encouraging preclinical findings, differences in irradiation parameters, experimental models, and study protocols continue to limit direct comparisons between studies. Therefore, further standardisation of experimental methodologies is needed to improve reproducibility and facilitate the translation of FLASH-RT into clinical practice. Continued development of robust and standardised research approaches will be crucial for understanding the mechanisms of the FLASH effect and supporting its future clinical implementation.

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