Advances in Radiation Oncology (2022) 8, 101124

advances
in radiation oncology

www.advancesradonc.org

Biology Contribution

# Ultrahigh-Dose-Rate Proton Irradiation Elicits Reduced Toxicity in Zebrafish Embryos

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Received 11 October 2022; accepted 8 November 2022

**Abstract**

**Purpose:** Recently, ultrahigh-dose-rate radiation therapy (UHDR-RT) has emerged as a promising strategy to increase the benefit/risk ratio of external RT. Extensive work is on the way to characterize the physical and biological parameters that control the so-called "Flash" effect. However, this healthy/tumor differential effect is observable in in vivo models, which thereby drastically limits the amount of work that is achievable in a timely manner.

**Methods and Materials:** In this study, zebrafish embryos were used to compare the effect of UHDR irradiation (8-9 kGy/s) to conventional RT dose rate (0.2 Gy/s) with a 68 MeV proton beam. Viability, body length, spine curvature, and pericardial edema were measured 4 days postirradiation.

**Results:** We show that body length is significantly greater after UHDR-RT compared with conventional RT by 180 $\mu$m at 30 Gy and 90 $\mu$m at 40 Gy, while pericardial edema is only reduced at 30 Gy. No differences were obtained in terms of survival or spine curvature.

**Conclusions:** Zebrafish embryo length appears as a robust endpoint, and we anticipate that this model will substantially fasten the study of UHDR proton-beam parameters necessary for "Flash."

## Introduction

The full potential of radiation therapy (RT) is currently limited by the dose absorbed by healthy tissues.[1] Recently, a benefit of ultrahigh-dose-rate (UHDR) irradiation with electron beams (UHDR-RT, >40 Gy/s), known as "Flash," has been reported.[2] UHDR-RT induced less lung fibrosis in mice compared with conventional dose rate RT (conv-RT) while preserving the antitumor efficacy. Several

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Sources of support: This work was supported by Inserm Cancer, the Ligue Contre le Cancer, IRC Transformed, Institut de Cancérologie de l'Ouest, the Stichting Tegen Kanker (STK) grant ZKD6219, and the Walloon region EPT grant D7.21214.014-P.

Disclosures: 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.

All the data are presented in the article and its supplemental materials. Specific requests can be addressed to the corresponding author.

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https://doi.org/10.1016/j.adro.2022.101124

studies have confirmed a protective effect of UHDR-RT in various animal models, tissues, and particles.[3] Combining the conformal precision of protons with UHDR-RT appears as a promising strategy to further reduce the toxicity of RT. In this respect, there is a strong methodological need to define the most favorable irradiation settings for "Flash."

The zebrafish embryo represents an appropriate model to study radiobiological effects such as viability and morphologic malformations.[4] Using UHDR electron beams, protection on zebrafish embryo length has been reported.[5,6] However, it is unclear whether proton beams behave similarly.[7] In this study, we assessed the biological response of zebrafish embryos in terms of survival, body length, spine curvature, and pericardial edema after 8 to 9 kGy per second 68 MeV proton RT.

## Methods and Materials

### Proton beam and dosimetry

Sixty-eight MeV protons were produced by an isochronous cyclotron (IBA Cyclone 70XP; IBA, Louvain la neuve, Belgique)[8]. A homemade pulsing chopper-based system allowed macropulses of controlled duration (>10 $\mu$s), frequency, and intensity.[9] This enabled conventional mean dose rates of 0.2 Gy per second up to UHDR of 9.2 kGy per second in identical conditions. Further details are given in supplementary Figs. E1 and E2, including online and film dosimetry.[10] Beam structures and doses are given in supplementray Tables E1 and E2.

### Zebrafish embryo maintenance and irradiation

Wildtype zebrafish (*Danio rerio*) embryos were cultured in E3 medium at 28°C in a humidified incubator (Memmert IN75, Schabach, Germany). One hour before irradiation, 32 eggs in 100 $\mu$l of E3 medium were placed in closed 0.7-mL Eppendorf tubes, prefilled with 0.5 mL solidified ultrapure agarose at 2 % (Invitrogen, Carlsbad, California) (Figs. E2, E4A). At 28 hours postfertilization (hpf), 30 and 40 Gy were delivered at 0.2 to 0.25 Gy per second (conv-RT) and 8.2 to 9.2 kGy per second (UDHR-RT) (Tables E1, E2). Two tubes were prepared for each condition and were irradiated with a homogeneous beam 10 mm in diameter. Three experiments were performed with strictly independent dates, egg batches, setup, and machine operation, except for Fig. 1 (2 replicates the same day). Viability rate (heartbeat) was assessed at 4 days postfertilization relatively to the number of surviving embryos 1 hour after irradiation. Pericardial edema was scored in living embryos according to previous report.[4] At 5 days postfertilization, embryos were fixed for 24 hours with 4% formol and photographed with a Ni-U stand and 2 × objective with 0.06 numerical aperture (Nikon Instruments, Melville, New York). The length (a) of embryos was measured along the vertebral column using Fiji software (ImageJ 1.53q). The distance (b) was measured by a straight line from the tip of the head to the end of the tail. Spine curvature represents the a/b ratio.

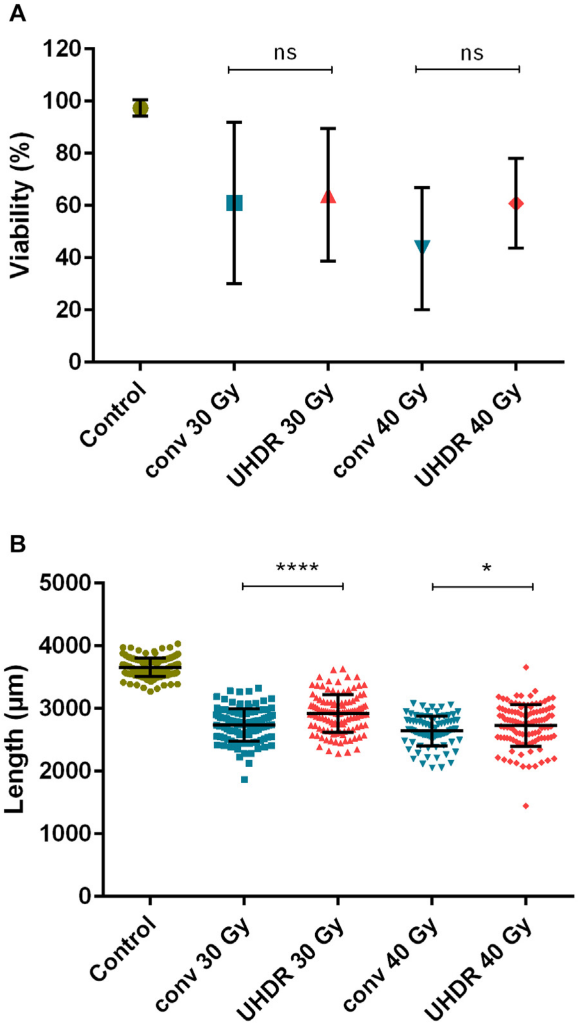

**Figure 1** Embryo development in response to ultrahigh-dose-rate (UHDR) proton radiation therapy versus conventional (conv) radiation therapy. A, Viability rate and B, length of zebrafish embryos at 4 d postirradiation (5 days postfertilization; n = 86-187/point). *Abbreviation*: ns = not significant.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 6). Kruskall-Wallis test followed by Dunn's correction was used for the mean of the survival rates. The mean values for the length, curvature, and pericardial edema were compared with a 1-way analysis of variance test followed by Bonferroni correction. Data represent the compilation of n = 55-187 ± standard deviation.

## Results

In preliminary experiments, the dose response of 4 and 28 hpf embryos was established to select the best conditions where RT affects development with little effect on viability (Fig. E3). Next, we tested whether irradiation dose rate influences zebrafish development. Viability of nonirradiated embryos was close to 100% (Fig. 1A). In contrast, irradiation led to decreased viability in all conditions with no statistical difference between conv-RT and UHDR-RT either at 30 Gy (64 ± 25% vs 61 ± 31%) or at 40 Gy (44 ± 23% vs 61 ± 17%). Next, body length of the embryos was assessed. A significant difference of 180 $\mu$m was observed between UHDR-RT (2920 ± 300 $\mu$m) and conv-RT (2740 ± 260 $\mu$m) at 30 Gy ($P < .0001$). To a lesser extent, zebrafish embryos irradiated at 40 Gy with UHDR-RT were also 90 $\mu$m longer (2730 ± 330 $\mu$m) than with conventional dose rate (2640 ± 240 $\mu$m; $P < .05$; Fig. 1B). The nonirradiated group exhibited the expected length of embryos at the early larval stage (3660 ±140 $\mu$m). Based on the body length reduction, toxicity after UHDR-RT was diminished by 20% at 30 Gy and 9% at 40 Gy. Notably, embryos irradiated at 40 Gy with UHDR-RT were as long as those irradiated with conv-RT at only 30 Gy. Interestingly, these observations are strongly dependent on the irradiation setup because a larger medium volume yielded negative results (Figs. E4, E5).

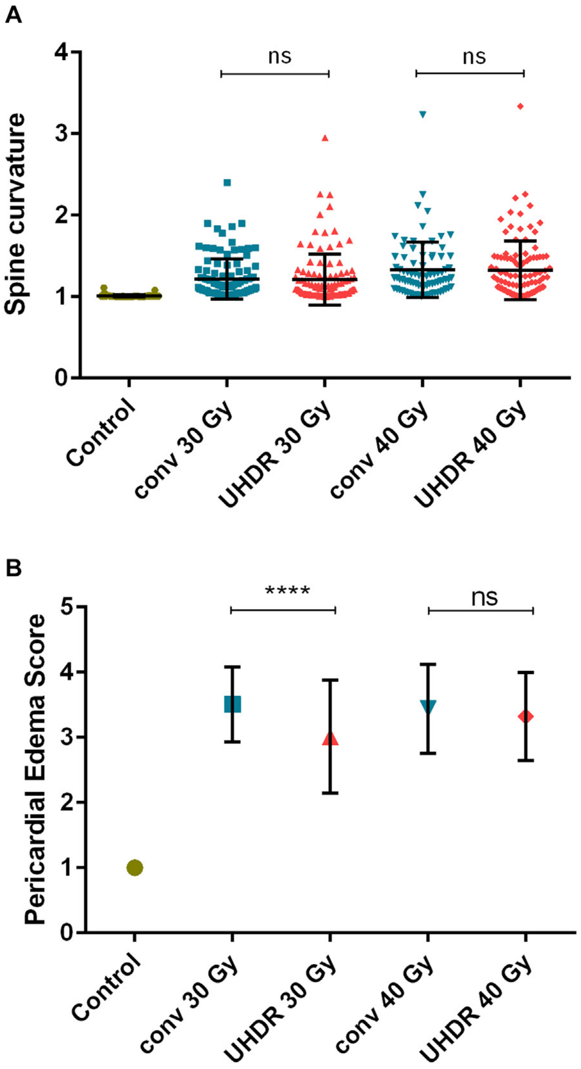

**Figure 2** Morphologic effects induced by ultrahigh-dose-rate (UHDR) proton radiation therapy versus conventional (conv) radiation therapy. A, Spine curvature value (length/Euclidean distance ratio). B, Pericardial edema score. (n = 83-113 embryos/point.). *** = p<0.001.

To further assess morphologic malformations, spine curvature and pericardial edema were investigated.[7] No curvature difference was observed between the UHDR-RT and conv-RT groups (Fig. 2A). No edema was observed for the control group (score = 1; Fig. 2B). Interestingly, a significant reduction was observed at 30 Gy UHDR-RT (score = 3.0) compared with conv-RT (score = 3.50), although not at 40 Gy.

Lastly, we investigated the influence of RT dose rate. Embryo length after 30 Gy RT was used as the most robust endpoint. Consistently with the precedent results, the highest dose rate (7700 Gy/s) led to 23% toxicity reduction compared with conv 0.2 Gy per second RT (Fig. 3). A trend was observed for groups >500 Gy/s although significance was not reached because of the low statistical power with repeated column comparison.

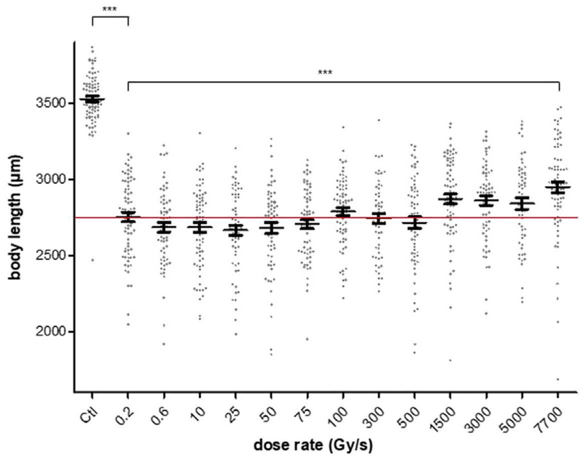

**Figure 3** Effects of dose rate on zebrafish embryo length. Length of zebrafish embryos at 4 d postirradiation (5 days postfertilization; n = 57-88/point). **** = $p<10^{-4}$, * = p<0.05. *Abbreviation*: ns = not significant.

## Discussion

The zebrafish embryo enables fast and quantifiable assessment of biological effects of irradiation beams with high statistical power.[4,11] In the present work, we investigated the effect of UHDR proton RT on pharyngulal stage 28 hpf zebrafish embryos. The radioresistance at 30 and 40 Gy agreed with a previous report.[12] A singular observation had initially reported the sparing of zebrafish embryo length using UHDR electron RT at an earlier stage (4 hpf) and lower dose (12 Gy).[5] Subsequently, an independent publication investigated zebrafish toxicity after UHDR proton RT, but hardly found conclusive results.[7] However, very recent work by the same group indicates that reduction of zebrafish toxicity with UHDR proton beams appears accessible.[13] In the present study, UHDR proton irradiation protected the length of the embryos. Pericardial edema was also reduced at 30 Gy. This is consistent with the publication by Karsch et al[13] that obtained protection with 30 Gy (300 Gy/s, 224 MeV) at 24 hpf but not with higher doses.[14] Collectively, these findings validate the use of the zebrafish model to study the biological effects of UHDR proton beams with varying energy or structure.

Other investigations used a $10^5$ Gy per second electron beam. Embryos irradiated at UHDR showed significant improvement in length and spinal curvature, and these effects were strengthened by reducing the $pO_2$[6]. Of note, the setup used here involves placing the embryos within a limited volume of medium and air. The fundamental role of oxygen level in response to UHDR proton RT appears as an interesting parameter in the prospect of future investigations.

## Conclusions

The present work validates the applicability of the zebrafish embryo length as a robust model for studying the toxicity of UHDR proton RT. This model provides a fast and reproducible readout that will further accelerate the establishment of the physical (beam structure) and radiochemical (oxygen involvement) parameters allowing to achieve the "Flash"-mediated protection of healthy tissues.

## Acknowledgments

We thank Professor Elke Beyreuther (University of Dresden) for helpful discussions, the Imagerie Pour Analyse de Contenu cellulaire facility, University of Rennes for zebrafish production, and Ms Nolwenn Dubois for technical help.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.adro.2022.101124.

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