



# First evidence of in vivo effect of FLASH radiotherapy with helium ions in zebrafish embryos

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1   **First evidence of in vivo effect of FLASH radiotherapy with helium ions in zebrafish**  
2   **embryos**

3

4   Y. Ghannam<sup>1\*</sup>, S. Chiavassa<sup>2,3\*</sup>, G. Saade<sup>1\*</sup>, C. Koumeir<sup>2,4</sup>, G. Blain<sup>2</sup>, G. Delpon<sup>2,3</sup>, M.  
5   Evin<sup>2</sup>, F. Haddad<sup>2,4</sup>, L. Maigne<sup>5</sup>, Q. Mouchard<sup>2</sup>, N. Servagent<sup>2</sup>, V. Potiron<sup>1,3</sup>, S. Supiot<sup>1,3</sup>

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7

8   **SUMMARY**

9         The ability to reduce toxicity of ultra-high dose rate (UHDR) helium ion irradiation has  
10      not been reported *in vivo*. Here, we tested UHDR helium ion irradiation in an embryonic  
11      zebrafish model. Our results show that UHDR helium ions spare body development and reduce  
12      spine curvature, compared to conventional dose rate.

13

14   **INTRODUCTION**

15         One challenge of radiotherapy (RT) is to effectively treat tumors while preserving  
16      healthy tissue. Preclinical evidence showed that UHDR radiotherapy selectively reduces the  
17      toxicity to healthy tissues, but not to tumors, as compared to conventional dose rate  
18      radiotherapy [1]. This "FLASH" effect, defined *in vivo per se*, has been mainly demonstrated  
19      using electrons. Recent data suggest that it also occurs with protons and hadrons [2,3]. The  
20      precise ballistics of the latter turn helpful when facing volumetric dose constraints [4].  
21      Combining this advantage with irradiation at UHDR is expected to further optimize the  
22      benefit/risk ratio. One study tested the Flash effect with helium ions at UHDR assessing only  
23      *in vitro* clonogenic survival [5]. However, there is currently no validation of helium ion UHDR-  
24      RT in complex organisms. In this study, we compared the developmental response of zebrafish  
25      embryos irradiated at UHDR or conv-DR using a helium ion beam.

1

2 **MATERIAL AND METHODS**

3 *Helium ion beam and experimental setup*

4 A monoenergetic  ${}^4\text{He}2+$  beam of 67.4 MeV was provided by the isochronous  
5 ARRONAX cyclotron (IBA Cyclone 70XP) [6] with a fixed micro-pulse frequency of 30.45  
6 MHz. A homemade pulsing chopper-based system allowed macro-pulses of controlled duration  
7 ( $>10\ \mu\text{s}$ ), frequency, and intensity [7]. This enabled conv-DR and UHDR in identical irradiation  
8 conditions. A 25  $\mu\text{m}$  tungsten foil and two collimators ( $\varnothing$  15 mm) were used to spread and  
9 homogenise the beam (Fig. 1A). Dose distribution and beam energy were evaluated by  
10 numerical simulation with Monte Carlo GATE [8]. The mean energy at the entrance of the  
11 sample was estimated to be  $40.3 \pm 2.3$  MeV, leading to a Bragg peak depth of 1.24 mm (Fig.  
12 1B). A plastic foil of 0.16mm was used to seal the wells and the embryos were maintained  
13 behind, on a single 1 mm thick layer (Fig. 1B and 1C). The mean Linear Energy Transfer (LET)  
14 inside the embryos was 27.6 keV/ $\mu\text{m}$ . Mean delivered physical doses were  $17.9 \pm 0.27$  Gy and  
15  $24.1 \pm 0.27$  Gy for UHDR and  $18.0 \pm 0.36$  Gy and  $24.0 \pm 0.29$  Gy for conv-DR. Considering a  
16 Relative Biological Effectiveness (RBE) between 1.5 to 2 [9], the theoretical biological doses  
17 were between 26.9 – 36.0 GyE and 36 – 48 GyE. A mean conv-DR of  $0.25 \pm 0.02$  Gy/s and  
18 UHDR of  $9.7 \pm 0.82$  kGy/s were applied. Beam structures, doses, online beam monitoring and  
19 dosimetry are presented in Table 1. Details on calculations are given in the Supplementary  
20 Material and Methods. Gray values (Gy) in the manuscript always refer to physical doses.

21

22 *Zebrafish embryo culture and irradiation*

23 Wildtype AB zebrafish (*Danio rerio*) eggs were provided at 1-hour post-fertilization  
24 (hpf) by the ImPACcell facility (Biosit, Univ. Rennes, France). Embryos were transported and  
25 cultured at 28°C in E3 medium. One hour prior to irradiation delivered at 28 hpf, 32 eggs were

1 placed as a single layer in 100  $\mu$ l (Fig. 1C). Viability rates were calculated on the 4<sup>th</sup> day  
2 relatively to the number of surviving embryos 1 hour after irradiation. At 5 days post-  
3 fertilization (dpf), cold-anesthetized embryos were fixed with 4% formol and later  
4 photographed with a Ni-U stand and 2X objective with 0.06 numerical aperture (Nikon  
5 Instruments, Melville, USA). The length (a) of embryos was measured by drawing along the  
6 vertebral column using Fiji software (ImageJ 1.53q). The Euclidean distance (b) was measured  
7 by a straight line from the tip of the head to end of the tail. Curvature represents the a/b ratio.  
8 Pericardial edema was scored as reported [10]. The toxicity reduction was calculated  
9 normalized to the conv-DR effect with the formula:  $100 * [1 - ((\text{ctl value} - \text{UHDR value}) / (\text{ctl}$   
10  $\text{value} - \text{conv-DR value}))]$ .

11 *Statistical analysis*

12 Three independent experiments (egg batch, beam, date) were performed to ensure  
13 reproducibility. GraphPad Prism (Version 6) was used for statistical analysis. Mean survival  
14 rates were tested with Kruskall-Wallis followed by Dunn's correction. Mean values of the  
15 length, curvature and pericardial edema were compared using a One-way Anova test followed  
16 by Bonferroni correction. For all figures, compiled data are presented  $\pm$  sd with \* =  $p < 0.05$ ; \*\*  
17 =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ .

18 **RESULTS**

19 To investigate the effect of UHDR  ${}^4\text{He}^{2+}$  ions, a homogeneous beam with real-time  
20 dosimetric control was designed at the Arronax cyclotron (Fig. 1A) for irradiating  $a \leq 1$  mm  
21 thick target in the plateau (Fig. 1B). Zebrafish embryos of 1240  $\mu$ m at 28 hpf were aligned  
22 vertically on the same plane using home-made well insert (Fig. 1C). Development and  
23 morphology of zebrafish embryos were measured after conv-DR and UHDR irradiations. As  
24 expected, viability of non-irradiated controls was close to 100% (Fig. 2A). No statistical  
25

1 difference was found between conv-DR and UHDR either at 18 Gy ( $87\% \pm 5$  vs  $94\% \pm 5$ ) or at  
2 24 Gy ( $80\% \pm 14$  vs  $90\% \pm 8$ ) (Fig. 2A). At 4 days post-irradiation, zebrafish embryos irradiated  
3 with 18 Gy UHDR had longer body length ( $3025 \pm 227$   $\mu\text{m}$ ) compared to those irradiated with  
4 conv-DR ( $2937 \pm 252$   $\mu\text{m}$ ,  $p < 0.001$ ) (Fig. 2B). Similar results were found at 24 Gy ( $2925 \pm 219$   
5  $\mu\text{m}$  vs  $2830 \pm 288$   $\mu\text{m}$ ,  $p < 0.0001$ ) (Fig. 2B). This represented a toxicity reduction by 15% at 18  
6 Gy and 14% at 24 Gy. Notably, embryos exposed to 24 Gy UHDR were as long as those  
7 exposed to 18 Gy conv-DR.

8 Embryos irradiated with both modalities developed a spine curvature, in opposite to  
9 controls. A significant reduction of the curvature ratio was observed at 24 Gy UHDR compared  
10 to conv-DR ( $1.10 \pm 0.19$  vs  $1.20 \pm 0.43$ ,  $p < 0.01$ ), although not at 18 Gy ( $p > 0.05$ ) (Fig. 3A).  
11 Similarly, pericardial edema was observed after RT but not in controls (Fig. 3B). The severity  
12 of edema was less important at 24 Gy ( $p < 0.05$ ) after UHDR (score =  $2.65 \pm 0.79$ ) compared to  
13 conv-DR (score =  $2.84 \pm 0.87$ ; (Fig. 3B).

14  
15 **DISCUSSION**  
16

17 The UHDR-mediated reduced toxicity has been demonstrated in various model  
18 organisms. With the zebrafish embryo model, protection in body length, pericardial edema or  
19 spinal curvature has been validated with electrons and protons, although not consistently [11-  
20 13]. Recent results from our laboratory confirmed a reduced toxicity of protons at UHDR [14].  
21 However, to our knowledge, no data has been published for helium ions *in vivo*.

22 The biological response to UHDR helium ion beams has been reported in human cancer  
23 cell lines *in vitro* with higher clonogenic survival only under 1% O<sub>2</sub> [5]. The present study  
24 shows for the first time the protective effect of UHDR helium ions *in vivo*. These results are  
25 consistent with our previous experiments using protons at similar beam energy and dose rates,  
26 leading to the same magnitude of protection by UHDR (toxicity reduction of protons: 9-20%

1 helium ions: 14-15%). It is nevertheless difficult to provide definitive conclusions regarding  
2 the biological doses, with effects of -25% to -28% in body size with conv-DR protons [14] and  
3 of -17% to -20% here. The present data fit better with a RBE in the lower estimated range, of  
4  $\approx 1.5$ . Some level of variability likely rises from varying orientation of the embryos and local  
5 oxygen gradients. The differential between conv-DR and UHDR lies between 100-200  $\mu\text{m}$ ,  
6 which is mostly in agreement with data from Beyreuther's group but not comparable with other  
7 studies performed at 4 hpf. Interestingly, helium ions are heavier particles than protons,  
8 resulting in higher linear energy transfer. High LET values are supposed to induce more  
9 efficient cell destruction, accompanied by reduced hypoxia dependence. Yet, the impact of the  
10 pO<sub>2</sub> on the FLASH effect in zebrafish has been demonstrated [13,15]. Our preliminary  
11 measurements suggest that eggs in our proton setup were more hypoxic (0-5 mm Hg) than those  
12 in the current helium setup ( $\approx 30$  mm Hg). Therefore, it would be interesting in future studies  
13 to perform experiments with helium ion beams in controlled O<sub>2</sub> environments in order to  
14 elucidate this apparent paradox.

15  
16 **CONCLUSION**  
17

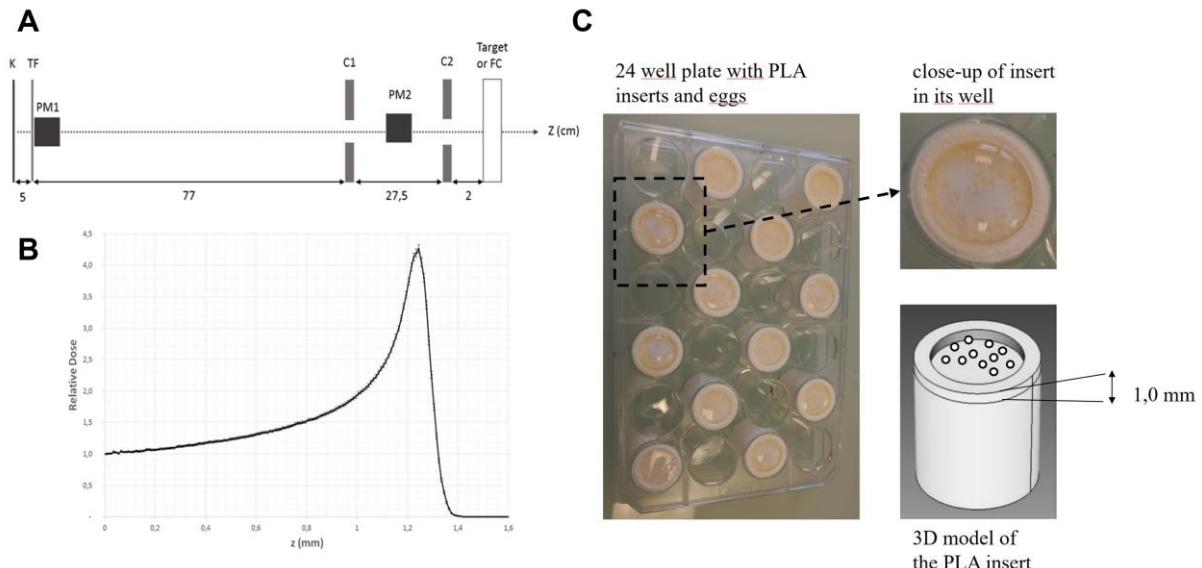
18 This study shows for the first time the *in vivo* effect of a UHDR helium ion beam. These  
19 findings advocate for the validity of combining high LET ion beams with the UHDR modality  
20 to benefit from both good ballistics and reduced toxicity.

21

Experiment	Macro-pulse Dose rate (Gy/s)	Mean Dose rate (Gy/s)	Macro-pulse width (ms)	Macro-pulse Frequency (Hz)	Number of macro-pulses	Total beam on time (ms)	Mean physical dose at the embryos (Gy)
FLASH day 1	7065	7065	2.05	-	1	2.05	14.8
	7255	7255	2.05			2.05	15.2
	7136	7136	2.74			2.74	20.0
	7052	7052	2.74			2.74	19.7
CONV day 1	7.23	0.23	1.1	28.6	1914	67 E+3	15.2
	7.03	0.22			1914	67 E+3	14.8
	7.23	0.23			2514	88 E+3	20.0
	6.85	0.22			2629	92 E+3	19.8
FLASH day 2	7734	7734	1.95	-	1	1.95	15.1
	7643	7643	1.92			1.92	14.7
	7758	7758	2.60			2.60	20.2
	7803	7803	2.60			2.60	20.3
CONV day 2	4.9	0.21	1.5	28.6	2029	71 E+3	15.0
	5.4	0.23			1829	64 E+3	14.7
	5.1	0.22			2571	90 E+3	19.9
	5.6	0.24			2457	86 E+3	20.5

1

2 **Table1. Beam structures and doses for conv-DR and UHDR irradiations.**



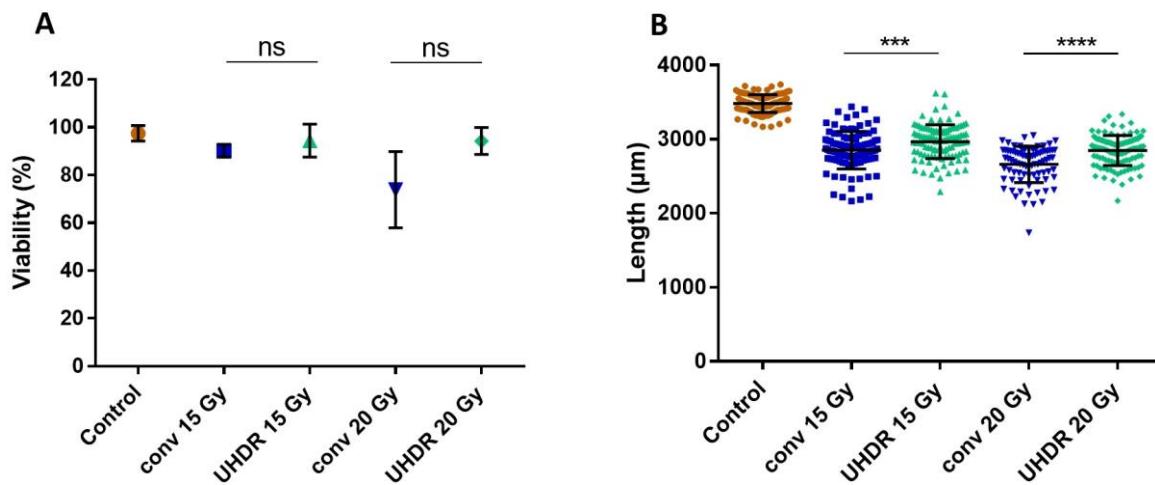
3

4 **Figure 1. Helium ion beam experimental setup.**

5 **A** Beam path with the exit kapton window (K), the tungsten foil (TF), the first collimator (C1),  
6 the photomultiplier tubes (PM1 and PM2), the second collimator (C2) and the Faraday cup  
7 (FC). Distances are in cm. **B** Bragg peak of the helium ion beam at the target level simulated in

1 water with GATE v9.0 [8]. The gray zone corresponds to the adhesive plastic foil for closing  
2 wells. **C** Zebrafish embryo positioning in the experimental target plate.

3



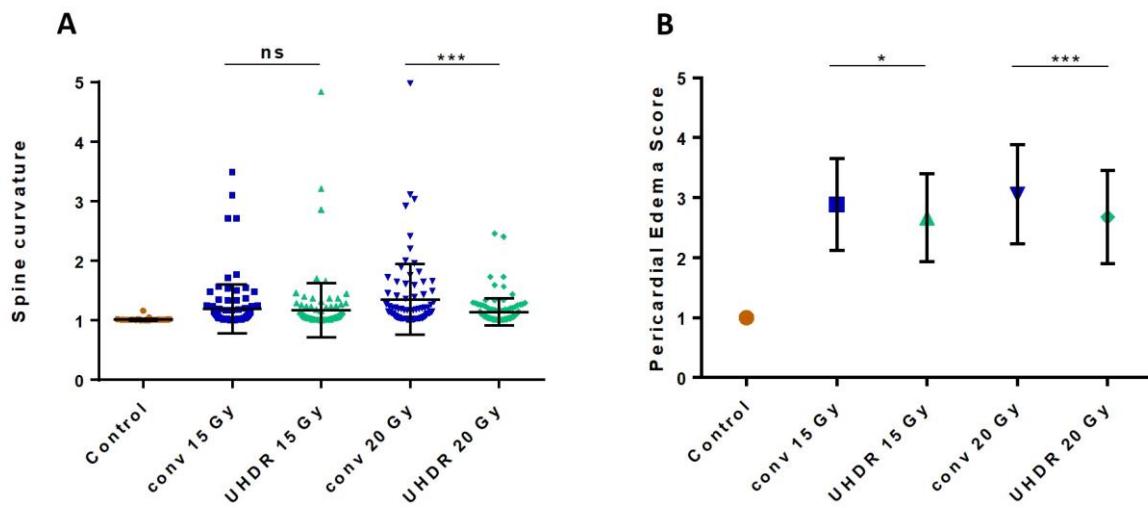
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6 **Figure 2. Effect of UHDR  ${}^4\text{He}2+$  irradiation on zebrafish embryo growth.**

7 Embryos were irradiated at 28 hpf. **A** Viability rate at 4 dpf. **B** Body length at 5 dpf. n= indicates  
8 the number of embryos per point.

9



10

11

12 **Figure 3. Effect of UHDR  ${}^4\text{He}2+$  irradiation on zebrafish embryo morphology.**

1 Embryos were irradiated at 28 hpf. **A** Spinal curvature (length/Euclidean distance ratio). **B**  
2 Pericardial edema score. n= indicates the number of embryos per point.

3

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13

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