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Electron dose rate and oxygen depletion protect zebrafish embryos from radiation damage

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Abstract

Background and purpose

In consequence of a previous study, where no protecting proton Flash effect was found for zebrafish embryos, potential reasons and requirements for inducing a Flash effect should be investigated with the beam pulse structure and the partial oxygen pressure (pO_2) as relevant parameters.

Materials and methods

The experiments were performed at the research electron accelerator ELBE, whose variable pulse structure enables dose delivery as electron Flash and quasi-continuously (reference). Zebrafish embryos were irradiated with ~26 Gy either continuously with a dose rate of ~6.7 Gy/min or in one 111 µs long pulse with a pulse dose rate of 10^9 Gy/s and a mean dose rate of 10^5 Gy/s, respectively. Using the OxyLite system to measure the pO_2 a low- ($pO_2 \leq 5$ mmHg) and a high-pO_2 group were defined on basis of the oxygen depletion kinetics in sealed embryo samples.

Results

A protective Flash effect was seen for most endpoints ranging from 4 % less reduction in embryo length to about 20 – 25 % less embryos with spinal curvature and pericardial edema, relative to reference irradiation. The reduction of pO_2 below atmospheric levels (148 mmHg) resulted in higher protection, which was however more pronounced in the low-pO_2 group.

Conclusion

The Flash experiment at ELBE showed that the zebrafish embryo model is appropriate for studying the radiobiological response of high dose rate irradiation. Pulse dose and pulse dose rate as important beam parameters were confirmed as well as the pivotal role of pO_2 during irradiation.

Introduction

Radiotherapy (RT) dose delivery techniques were continuously improved during the last decades with respect to tumor conformity. However, the still unavoidable exposure of normal tissue holds the risk of severe side effects, which negatively affect patient's quality of life. Strict dose constraints, but also the application of charged particles with its beneficial inverse dose profile are existing options to protect normal tissue. Alternatively, the recently described Flash effect [1], i.e. the delivery of therapeutic doses at high dose rates within maximal 500 ms, promises better normal tissue protection but similar tumor treatment efficacy compared to conventional, continuous dose delivery over minutes. The protective effect of very high dose rates was primarily shown for electron Flash, and in the following verified for electrons and photons revealing less normal tissue side effects in different species [2–6]. Summarising the published Flash studies, a recipe for Flash-RT [7] was formulated recommending mean dose rates of 100 Gy/s, pulse dose rates of ~10^6 Gy/s, and minimum doses per pulse (> 1 Gy) and fraction (> 10 Gy). Moreover, it is assumed that the oxygen concentration of the irradiated tissue is important for the presence of a Flash effect [5, 7–9].

Several attempts were made at clinical proton facilities [10–12] to verify a protecting proton Flash effect. Exemplarily, Diffenderfer et al. [12] have shown that high proton dose rates significantly reduce the radiation damage in the intestine of mice, whereas flank tumors of these mice were treated efficiently. By contrast, the irradiation of zebrafish embryos at the same type of cyclotron failed to reveal an influence of proton dose rate on radiation induced morphological alterations [11]. In the discussion of this experiment, three potential explanations were identified for the missing Flash effect: 1) the zebrafish model itself; 2) a pulse-time-regime that did not fulfil the recommendation by [7] and 3) an uncertain oxygen level during irradiation that might mask a high dose rate effect [5, 7, 9, 13, 14].

In order to study the influence of 1) – 3) an experiment was scheduled at the research accelerator ELBE (Electron Beam of high Brilliance and low Emittance, [15]) at HZDR. ELBE provides an electron beam of highly variable pulse structure [16, 17] that was deployed in the present study to mimic the conventional, quasi-continuous dose delivery of a clinical Linac ("reference") and to generate a ~0.1 ms long electron pulse of maximum pulse dose rate of 10^9 Gy/s. At this ultra-high

pulse dose rate a Flash effect should be observable in zebrafish embryos referring to a previous study with pulse dose rates of 10^5 Gy/s [2]. The influence of oxygen was studied by irradiation of embryos under controlled conditions at high or low partial oxygen pressure (pO_2).

Materials and methods

Experimental setup and control of dose delivery at the ELBE accelerator are described in the Supplement. For the present experiment, a previously established setup [16–18] was complemented by additional measurement devices for online control of dose delivery.

Embryo handling and oxygen measurements

Zebrafish embryos were treated as described previously (Supplement, [19]), except irradiation and oxygen deprivation in 0.5 ml Eppendorf tubes. The tubes were filled with 200 µl low-melting agarose (Fig. S2b, UltraPure® Agarose, Invitrogen, Germany) to assure comparable sample height and to protect embryos from shear forces in the narrow tip of the tube. For irradiation, about 30 embryos (25-40) were placed in the tubes, which were filled with E3 embryo medium [20] and enclosed.

The partial oxygen pressure was determined in E3 surrounding the embryos in the enclosed Eppendorf tube using the OxyLite™ (Oxford Optronix Ltd, Abingdon, UK; [21]) system. Oxygen depletion kinetics were measured by inserting the sensor in a tube with a small hole in the cap, which was sealed with Parafilm to avoid gas exchange during measurement. In consequence of previously measured kinetics (Fig. 1) the embryos were treated in two groups: a low-pO_2 group, which was maintained in sealed tubes one hour prior irradiation, and a high-pO_2 group, treated as fast as possible after sealing. Actual kinetics were proven by daily measurements in parallel to sample irradiation.

Endpoints

Three independent experiment replications were performed on consecutive days using embryos of different breeding pairs. Applying similar observation periods for all samples, embryonic survival and morphological alterations, like pericardial edema (pe) and curved spines (sc) were assessed daily over the four-day follow up period. Morphological alterations were related to the actual numbers of surviving embryos, whereas the survival itself was related to the number of living embryos at

irradiation day. In addition to that, the severity of pericardial edema ($SV_{mean,PE}$) and spine curvature ($SV_{mean,SC}$) was assessed from pictures taken at the 4th day post-irradiation (dpi) [19] by staging from 1 (normal appearance) to 4 (most severe damage). Embryo body length and diameters of eye and yolk sac were also determined in these pictures (Fig. S2c) using the software ZEN (Version 2.6, Zeiss, Germany). The severities as well as the length and diameters were determined as mean values per sample, i.e., averaged over all surviving embryos.

Statistical analyses

Two-sample two-sided t-tests of independent samples were performed to compare the endpoints between reference and Flash irradiation and between Flash subgroups (irradiation before vs. after reference). Correlations between the endpoints and the most important experimental parameters (applied dose, sealing time (i.e. pO_2), irradiation time, experiment repetition (day, one-hot-encoded)) were evaluated using the Pearson correlation coefficient R to check for dependencies. To confirm that the impact of Flash irradiation was independent of these experimental parameters, multivariable linear regression was additionally performed. Every endpoint that showed a significant difference between reference and Flash in the t-test was individually considered as the dependent variable, while applied irradiation (reference or Flash) and the four experimental parameters were simultaneously included as independent variables. All analyses were performed with SPSS 25 (IBM Corporation, Armonk, NY, USA) and p-values below 0.05 were considered as statistically significant.

Results

External influences on the radiation response

Comparability of experiment parameters, like irradiation and sealing time, for reference and Flash regime was confirmed by the non-significant results of the t-test ($p>0.45$). Likewise, a dose homogeneity better than 90 % over the irradiation field (width 6.5 mm, height 3 mm) and an uncertainty of 10 % for the absolute dose, mainly caused by the uncertainty of radiochromic film calibration, was achieved for samples irradiated at both regimes. A statistically significant difference ($p=0.013$) was, however, found for the mean doses (± spread) of 26.2 ± 1.4 Gy for reference and

26.7 ± 1.6 Gy for Flash irradiation, respectively, which corresponds to a dose difference of 2 %. Considering the treatment dose, this small difference was of no (radiobiological) consequence, but confirms the reproducibility of dose delivery over all experimental days. Adverse effects of volume restriction, temporal hypoxia and experiment conditions on embryonic radiation response were excluded by comparing the control samples running in parallel to the treatment and those remaining in the lab, which exemplarily resulted in similar mean lengths (Table S1).

The daily measured oxygen data (Supplement) were summarized in a common oxygen deprivation kinetics, which was applied to estimate the actual pO_2 prevailing at irradiation time in the embryo medium on basis of the sealing time before treatment (Fig. 1). Independent on treatment group, the potential influence of varying sealing times, i.e. actual pO_2 at irradiation, was considered in the further analysis as one experimental parameter.

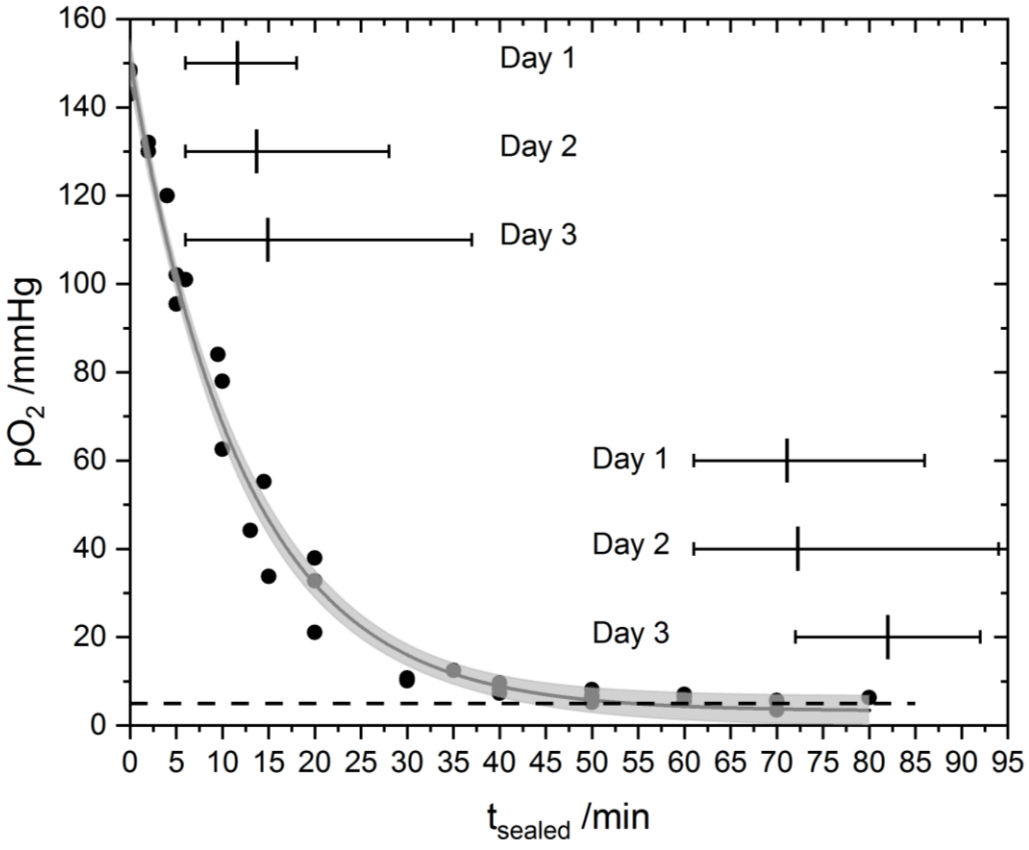

Fig. 1: Decline of pO_2 after sealing of the embryos in an 0.5 ml Eppendorf tube. Assuming exponential decay the daily measured pO_2 values (black dots) were fitted to pO2 kinetics (grey line, $pO_2=147.04\times exp(-t_{sealed}/12.26)+3.26$, Supplement). The 95% confidence interval is given in light grey. The range of sealing times for the high and low pO_2 group of each experimental day is indicated by the line segments for the respective range of times. Mean values of each subgroup are marked by vertical lines; the level of radiobiological hypoxia (5 mmHg) is indicated by the horizontal, dashed line.

Comparison of Flash and reference

From the 98 samples (62 samples Flash, 36 reference) irradiated at ELBE, two Flash treated samples had to be excluded due to accidental overdosage. The Flash regime turned out to be beneficial for most of the endpoints (two-sample t-test; Table 1), except yolk sac diameter and embryonic survival observed at the 4th dpi. Indeed, the survival was higher after reference irradiation, but since the overall embryonic survival after both treatments was not distinguishable from the controls (96.06 ± 4.00) this endpoint was excluded from further analysis. Moreover, contrary to later time points, no significant differences between the two regimes were observed at the 2nd dpi. This might indicate the temporal development of morphological malformations, since almost all embryos were hatched and clearly visible at that time. For the other endpoints, a significant protecting Flash effect was found ranging from almost 25% less embryos with spinal curvature to 4% longer embryos and larger eyes at 4 dpi. Notably, stable reductions of about 20 % and 25 % were seen in the number of embryos with pericardial edema and spinal curvature from the 3rd dpi on.

Table 1: Comparison of the mean values ± standard deviation (sd) of the different endpoints analysed in zebrafish embryos irradiated with Flash and reference electron regime. P-values of the t-test are given in the last column. Embryo length, diameters of yolk sac and eye as well as the severities of pe an sc were determined on basis of the pictures taken from all embryos at the 4th dpi.

Endpoint	Flash ± sd	Reference ± sd	p-value
Survival /%	96.67 ± 3.22	98.23 ± 2.44	0.014
PE 2dpi /%	42.73 ± 27.72	47.12 ± 29.12	0.464
PE 3dpi /%	49.20 ± 27.44	62.78 ± 25.50	0.018
PE 4dpi /%	65.65 ± 26.50	81.33 ± 17.89	0.002
$SV_{mean,PE}$	1.95 ± 0.50	2.29 ± 0.42	0.001
SC 2dpi /%	40.97 ± 26.92	47.38 ± 27.62	0.266
SC 3dpi /%	44.25 ± 28.04	57.39 ± 29.05	0.031
SC 4dpi /%	36.72 ± 21.46	48.89 ± 21.81	0.009
$SV_{mean,SC}$	1.59 ± 0.41	1.77 ± 0.41	0.041
Length /µm	3743.42 ± 189.63	3599.67 ± 155.19	<0.001
$Diameter_{Yolk}$ /µm	500.64 ± 23.67	507.47 ± 18.56	0.144
$Diameter_{Eye}$ /µm	212.88 ± 12.56	205.03 ± 11.40	0.003

dpi…day post irradiation, PE…pericardial edema, sc…spinal curvature, SV..severity

The weak correlation to radiation dose (|R|<0.2) was confirmed on endpoint level by the Pearson correlation coefficient R (Table S2). In addition, moderate correlations to sealing and irradiation time and a strong correlation to experiment day were revealed for most of the endpoints.

Taking into account these influences, a protecting effect of high electron dose rate was still revealed by multivariable linear regression (Table 2). Both, the number and the severity of morphologic malformations were significantly reduced after Flash compared to reference irradiation ($p<0.05$). Likewise, the embryos were longer (Fig. 2a) and the eye diameter was larger in Flash samples ($p<0.05$). Irradiation and sealing time as analogies to embryo age and remaining pO_2 level followed in general the same dependencies: the older the embryo and the longer the sealing the less damage was induced and the stronger the protecting effect by Flash (Fig. 2b, c). Surprisingly, most endpoints were also strongly related to the irradiation day, exemplarily shown in Figure 2d for the endpoint embryo length, although independent of the protecting Flash effect. The radiosensitivity or the strength of the radiation effect seemed to increase over time, which is not explainable by biology, since for each day embryos of several clutches were mixed. Moreover, experimental timing, room temperatures and procedures were similar for all days. The broadening of the sealing time window (Fig. 1) and the consequential broader pO_2 distribution for the high-pO_2 group as well as the increased workload in the lab due to follow up in parallel to experiment are potential sources of this effect. The protecting effect of high dose rate electron irradiation becomes evident under all circumstances, but most pronounced for the low-pO_2 group (Fig. 2c).

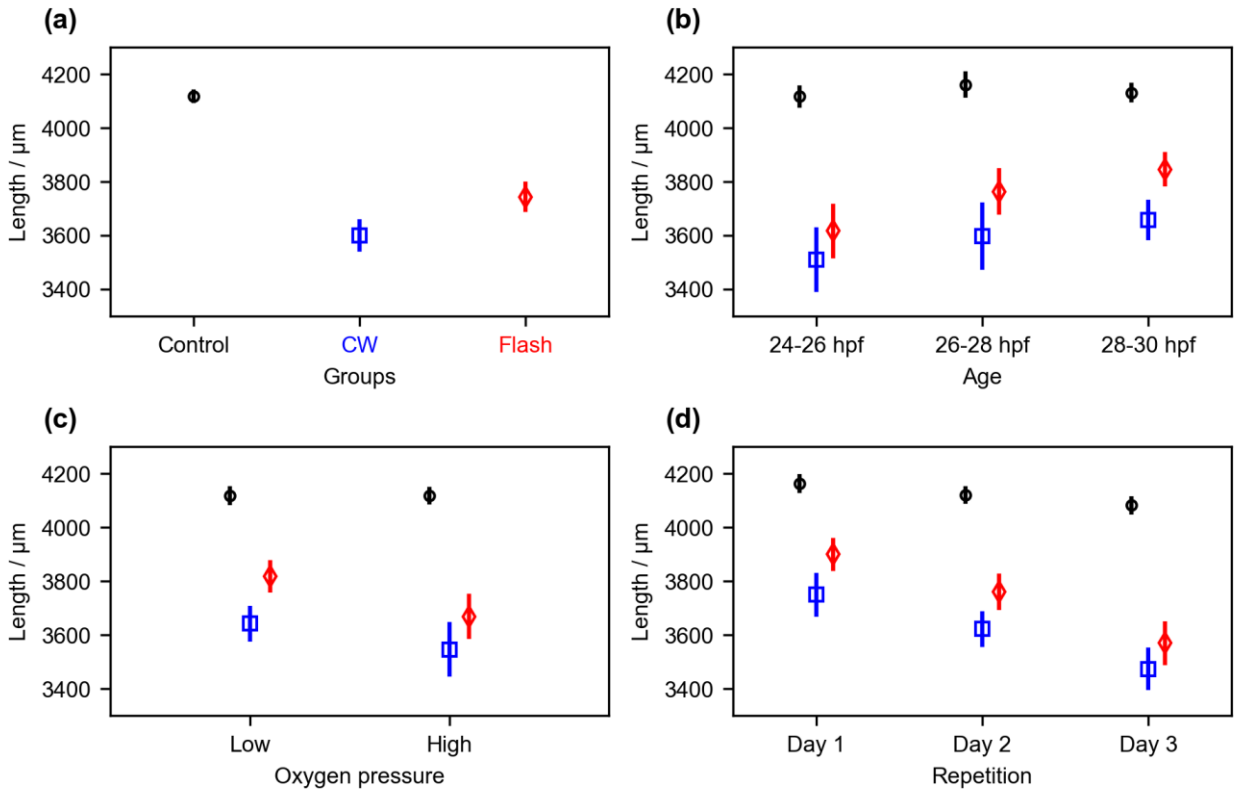

Fig. 2: Subclassification of the treatment groups reference (blue square), Flash (red diamond) and control (black dot) to elucidate the influence of the different experiment parameters. The mean lengths and 95% confidence intervals are given for whole treatment groups (a) and the subgroups formed according to irradiation time and consequential embryo age (hpf, hours post-fertilization) (b), partial oxygen pressure at treatment (c) and experiment repetition (d).

Two-sample t-tests for all endpoints between the two Flash subgroups were finally applied to check for any bias from the fixed sample order (Flash – reference – Flash) within each run that results in different sealing times and consequentially different pO_2 (Table S3). Significant differences were found for the embryo length, for the spinal curvature measured at the 3rd and 4th dpi and for the severity of the induced morphological malformations. Thereby, less damage was indicated for those samples treated after the reference-sample, most likely due to the further reduction in pO_2.

Table 2: Fit parameters and coefficient of determination (R) returned from the multivariable linear regression of different endpoints and the respective experimental parameters. For each endpoint the fitted parameters (upper row), their standard deviations (middle row) and the significance (p-values, lower row) are given. Significant parameters are bold labelled.

Endpoint	R	Flash (0:Reference, 1:Flash)	Dose /Gy	Irradiation time	Sealing time /min	2nd exp day (baseline: 1st day)	3rd exp day (baseline: 1st day)	Constant
PE 3 dpi /%	0.840	**-12.997**	0.603	**-0.050**	**-0.312**	**20.256**	**46.967**	47.514
		3.390	2.004	0.018	0.054	4.528	3.955	53.784
		<0.001	0.764	0.007	<0.001	<0.001	<0.001	0.379
PE 4 dpi /%	0.869	**-15.335**	0.396	**-0.063**	**-0.258**	**17.218**	**41.429**	74.952
		2.791	1.650	0.015	0.045	3.727	3.256	44.277
		<0.001	0.811	<0.001	<0.001	<0.001	<0.001	0.094
$SV_{mean,PE}$	0.898	**-0.331**	0.004	**-0.001**	**-0.007**	**0.293**	**0.793**	**2.360**
		0.050	0.030	0.000	0.001	0.067	0.058	0.792
		<0.001	0.89	<0.001	<0.001	<0.001	<0.001	0.004
SC 3 dpi /%	0.837	**-11.180**	-0.528	-0.034	**-0.244**	**19.997**	**53.582**	62.673
		3.625	2.143	0.019	0.058	4.842	4.230	57.514
		0.003	0.806	0.077	<0.001	<0.001	<0.001	0.279
SC 4 dpi /%	0.857	**-12.613**	-0.934	**-0.104**	**-0.251**	**12.318**	**26.265**	**93.509**
		2.624	1.551	0.014	0.042	3.504	3.061	41.627
		<0.001	0.549	<0.001	<0.001	0.001	<0.001	0.027
$SV_{mean,SC}$	0.850	**-0.183**	-0.026	**-0.002**	**-0.005**	**0.228**	**0.500**	**2.828**
		0.050	0.030	0.000	0.001	0.067	0.059	0.796
		<0.001	0.383	<0.001	<0.001	0.001	<0.001	0.001
Length /µm	0.912	**141.713**	-1.953	**0.642**	**1.621**	**-144.427**	**-321.228**	**3607.252**
		17.747	10.494	0.093	0.284	23.704	20.708	281.580
		<0.001	0.853	<0.001	<0.001	<0.001	<0.001	<0.001
D_{eye} /µm	0.763	**8.576**	**-2.531**	-0.001	**0.119**	0.678	**-15.979**	**271.950**
		1.870	1.106	0.010	0.030	2.497	2.182	29.666
		<0.001	0.024	0.912	<0.001	0.787	<0.001	<0.001

dpi…day post irradiation, PE…pericardial edema, sc…spinal curvature, SV..severity, D_{eye}.. eye diameter

Discussion

The combination of the beneficial effects of high dose-rate Flash-RT and proton depth dose distribution promises the differential sparing of normal tissue under similar tumor treating efficacy. Several attempts were made to study the influence of high proton dose rate *in vitro* [22–26] and *in vivo* [12, 27, 28], whereof just a few experiments have revealed a normal tissue protecting effect [12, 22, 24]. For example, no effect of proton dose rate was obtained in a previous study on zebrafish embryos [27]. In consequence of this experiment, the present work was scheduled to study the zebrafish embryo model, the beam pulse structure and the partial oxygen level as potential reasons for the missing Flash effect using electron beams.

The zebrafish embryo model as alternative vertebrate model is more and more applied in radiobiology and preclinical research in general [19, 29]. Intermediate between *in vitro* culture and rodent, the model was used to prove the protecting effect of electron Flash [2] and to study the formation of reactive oxygen species as one of its mechanisms [5]. The sole difference between the electron [2] and the proton Flash [27] experiment was the embryo age of 4 hours post-fertilization (hpf) for the first and 24 hpf for the latter. Zebrafish embryos become more radioresistant with age [30], for what reason a dose of 26 Gy, instead of 8 Gy like in the electron studies, was applied in the present work. This dose was necessary to induce measurable morphological alterations but did not significantly reduce embryonic survival. Moreover, the influence of embryo age during the 6-8 hour irradiation of the samples was considered by alternating irradiation of both regimes and is visible by the increasing embryo length with increasing age (Fig. 2b). The distribution of embryo age was comparable for both groups and does not alter the outcome of the present study (Table 3). Consequently, the zebrafish embryo model is applicable to study the Flash effect on a whole organism level provided that the age is taken into account and that the required doses are achieved.

The pulse dose rate was another parameter that clearly distinguishes the zebrafish proton Flash experiment [27] from previous electron studies [2, 7]. Although the mean dose rate of about 100 Gy/s was reached in all experiments, maximal proton pulse dose rates of 10^3 Gy/s [27] were available

considerably lower than the 10^5-10^6 Gy/s recommended for electron Flash [7]. Different from that, the ELBE electron beam provides pulse dose rates of 10^9 Gy/s and time averaged dose rates of 10^5 Gy/s that clearly exceeds the parameters recommended for Flash [7]. Applying these parameters, a protective Flash effect was seen for the majority of endpoints ranging from 4 % less reduction in embryo length and eye diameter to about 20 – 25 % less embryo with spinal curvature and pericardial edema at final observation day, relative to the reference irradiation. This result is of similar magnitude like the 20 % improvement in neurocognitive performance [3] and reduction of lung fibrosis [1] after treating mouse brains and thorax with electron Flash instead of conventional dose rates. In contrast to the present finding, in previous *in vitro* studies at ELBE the radiation response was not altered by pulse dose rates of 10^9 Gy/s [16, 17], which indicates that this factor alone is not sufficient for an improved response by electron Flash. Likewise, in most of the *in vitro* experiments with laser-driven particles no influence of the inherent ultra-high pulse dose rates on cellular outcome could be revealed [23, 24, 26, 31–33]. Most probably, low pulse doses [7, 8], the focus on tumor cells and the atmospheric partial oxygen level *in vitro* are responsible for the missing dose rate effect.

The partial oxygen level of irradiated tissue as a critical factor for ultra-high dose rate effects was already mentioned decades ago [34–37] and also pointed out in the context of Flash-RT [5, 7–9, 13]. Flash or any other high dose rate effects seem to require physoxia (20 – 50 mmHg) or hypoxia (<5 mmHg) in the irradiated sample [38] and a sufficient pulse dose to deplete enough oxygen in the irradiated volume [7, 8, 13, 36]. In a simulation study [8], it was shown that pulse doses of 10 Gy and higher are necessary to alter the radiation response in physoxic samples. Under atmospheric oxygen pressure (150 mmHg) the required pulse doses for oxygen depletion approach few 100 Gy [8], which is incompatible with cellular or animal survival. An inappropriate partial oxygen level was assumingly a main reason for the missing proton Flash effect in the previous study [27]. There, measurement of pO_2 was not performed and the embryo dwell time in the sample holder was minimized to avoid potential adverse effects for the embryos. In the present work, the oxygen consumption was characterized and its kinetic (Fig. 1) was utilized to treat the embryos at different pO_2. For most of the endpoints, the Flash regime was protective under physoxia (high-pO_2) as well as radiobiological hypoxia (low-pO_2). The pronounced effect in the low-pO_2 group (Fig. 2c) seem to be incompatible

with the idea, that hypoxia would prevent the protecting Flash effect by already low oxygen levels [7] and raises the question if the Flash effect also occurs in tumors. However, since direct pO_2 measurement in the embryos was not possible without killing them, the surrounding medium was used as surrogate keeping in mind that the chorion of the embryos might act as a buffer for quite some time. During the experiment, no oxygen deficiency effects were observed in the controls for sealing times ≤100 minutes, which somehow supports this hypothesis.

From the present study, but also from literature, it is not clear if oxygen depletion is the only biological factor that determines the Flash effect. Other factors like the influence of the immune system [39] and an altered response of stem cell niches [13] are also discussed. However, to study these issues more complex organisms are required that allow for long-term follow up and detailed radiobiological studies on individual organs. For the parameters considered in the present study, like embryo age, an influence on the radiation response in general was seen, which has however no impact on the protecting effect of high dose rate electron treatment. The surprisingly observed day-to-day variation of the radiation effect could not be clarified, but indicates statistical variations in the radiosensitivity of different embryo batches or the influence of workload on individual experimental days. More important, these variations pointed out the general necessity of repetitive experiments on several days.

In conclusion, the Flash experiment at ELBE showed that the zebrafish embryo model is appropriate for the investigation of the radiobiological response of high dose rate irradiation. The high doses required to induce a Flash effect in 24 hpf embryos might be challenging for some accelerators, whereas the study of individual organs might be tricky from a technical point of view. The importance of the partial oxygen pressure in the irradiated sample was confirmed by a clear Flash effect below atmospheric pO_2. In order to further clarify the influence of pO_2, a repetition of the zebrafish proton Flash experiment under more controlled pO_2-conditions in the embryos would be necessary.

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