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Pulse structure and dose rate as determinants for the Flash effect observed in zebrafish embryo

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Highlights ((maximum 85 characters, including spaces, per bullet point).**max 5 bullet points)**

- Mean dose rate is the determinant factor for inducing electron Flash effect
- Isochronous and synchrocyclotron pulse structures enable proton Flash effect
- Proton Flash effect in zebrafish embryo confirmed at isochronous cyclotron

Abstract. The abstract should not exceed 250 words.

Background and purpose

Continuing recent experiments at the research electron accelerator ELBE at the Helmholtz-Zentrum Dresden-Rossendorf the influence of beam pulse time structure on the Flash effect should be investigated in a zebrafish embryo model.

Materials and methods

The pulse structures of an isochronous and a synchrocyclotron were mimicked at ELBE with mean dose rates of 287 Gy/s and 177 Gy/s and pulse dose rates of 10^6 Gy/s and 10^9 Gy/s, respectively; and a macro pulsing for the latter. For comparison, a maximum (mean dose rate 2.5 x 10^5 Gy/s, pulse dose rate ~10^9 Gy/s) and a reference (mean dose rate of ~8 Gy/min) regime were applied. Radiation induced changes were assessed in zebrafish embryos over four days post irradiation.

Results

A significant protecting Flash effect with a clear dependence on mean dose rate was revealed for almost all endpoints and all electron pulse regimes relative to the reference. The macro pulse dependent prolongation of treatment time of the synchrotron-like regime reduce the protecting effect compared to the maximum regime delivered at same pulse but higher mean dose rate. The protecting Flash effect of the cyclotron-like regime was confirmed at a clinical isochronous proton cyclotron comparing the effects induced by 300 Gy/s relative to conventional proton beam delivery.

Conclusion

The mean dose rate or treatment time are more important than pulse dose rate for the extent of the normal tissue protecting Flash effect.

Introduction

The Flash effect describes the radiobiological observation of normal tissue sparing for doses with efficient tumor killing by ultra-high dose rate (UHDR) irradiation [1–3]. Firstly demonstrated

for electrons [1], the Flash effect was in the meantime shown for all clinical relevant types of radiation [2, 4, 5] using radiobiological models of different levels of complexity. Most of the Flash effect studies used electron beams delivered by clinical-like linear accelerators (linacs) with macro pulse structure (microsecond pulse duration, millisecond pulse interval). However, only few experiments analyzed the impact of pulse structure and dose rate of the applied accelerators [4, 6]. One systematic study on mouse brains [6] identified a mean dose rate of 100 Gy/s as a lower electron dose rate limit for a neuroprotective effect, which was later on extracted as one of the minimal requirements for inducing an electron Flash effect in some topical reviews (e.g. [2, 7, 8]). Other parameters, like maximum beam delivery times of 100 ms and pulse dose rates $>10^5$ Gy/s are under discussion.

For protons, most experimenters have to rely on clinical machines, i.e., isochronous and synchrocyclotrons, and synchrotrons with their given beam pulse structure [4, 9, 10]. The differences in beam delivery from quasi-continuous (isochronous cyclotron) to macro pulsing (synchrocyclotron) to beam extraction in spills (synchrotron) complicates the analysis of the available proton Flash experiments regarding common beam requirements analogue or distinct to electron Flash irradiation [3].

To understand the influence of the beam pulse structure and eventually identify the most preferable one with respect to normal tissue protection, systematic studies on the radiobiological effect of UHDR irradiation, independent on LET, are necessary [10, 16]. The ELBE research electron accelerator (Electron Beam of high Brilliance and low Emittance, [17]) at the Helmholtz-Zentrum Dresden-Rossendorf (HZDR) with its variable pulse structure and beam intensity is the ideal tool for this purpose. In continuation of previous experiments [14, 18], the ELBE beam was deployed in this study to mimic the beam pulse structure of three different UHDR irradiation regimes that reflect the maximal available pulse dose rate ($UHDR_{max}$) at ELBE, the pulse structure of a clinical isochronous cyclotron ($UHDR_{iso}$) and of a clinical synchrocyclotron ($UHDR_{synchro}$). Moreover, a quasi-continuous reference beam of conventional dose rate (Figure S1) was generated. The radiobiological effects of these four

pulse regimes were assessed in wildtype zebrafish embryos [18, 19]. Finally, the observed Flash effect for the $UHDR_{iso}$ regime was confirmed by proton irradiation at a clinical isochronous cyclotron.

Materials and Methods

Electron irradiation

The superconducting electron linear accelerator ELBE [17] provided beams of 30 MeV electron bunches (13 MHz basic bunch frequency, 5 ps bunch length) that were modulated to achieve the different beam pulse structures (Table 1). For the $UHDR_{iso}$, the $UHDR_{max}$ and the reference regime the electron bunches were delivered quasi-continuously at 13 MHz frequency, but differ in bunch dose by several orders of magnitude resulting in different irradiation durations. In contrast to the quasi-continous regimen, the treatment dose of the $UHDR_{synchro}$ regime is delivered by five macro pulses at a macro pulse frequency of 25 Hz. Each of these macro pulses contain ~800 electron bunches which were emitted within 40 ms, which sums up to a macro pulse dose rate of 0.92×10^5 Gy/s. The irradiation setup and dosimetry are applied as described in [18] and summarized in Supplement S1 and S3.

Proton irradiation

Proton irradiation was realized at the horizontal fixed-beam beamline in the experimental hall of the University Proton Therapy Dresden (UPTD, [14]). Embryos were irradiated with 224 MeV proton beams (linear energy transfer, LET = 0.417 keV/µm) using a beam current of 150 nA for high dose rate delivery of ~300 Gy/s and 0.07 nA for reference irradiation of 0.15 Gy/s, respectively. Setup and dosimetry are described in Supplements S2 and S3.

Embryo handling and endpoints

Four independent experiment replications were performed at consecutive days applying a timed procedure [18] starting with transport of wildtype AB zebrafish embryos from the Center for Regenerative Therapies TU Dresden (CRTD) to the ELBE or UPTD facility followed by maintenance at room temperature (23 – 24 °C). Approximately 1 h before irradiation about 30

embryos were sealed in 0.5 ml Eppendorf tubes each filled with 200 µl low melting agarose (UltraPure® Agarose, Invitrogen, Germany) and ~ 300 µl E3 embryo medium [20] (referred to one ”sample“ hereafter). At ELBE, the irradiations were performed in up to 11 runs per day, which last approximately 6 hours, i.e. with embryos of 24 to 30 hours post-fertilization (hpf). Accordingly, the experiment at UPTD involve 4 - 7 runs per day and embryos of 24 to 29 hpf. The influence of embryo ageing during irradiation was minimized by application of all regimes, four for electrons and two for protons, in each run. An alternating order of irradiation regimes was used to circumvent the impact of sealing times. The time for oxygen consumption as well as its influence on the embryos was controlled at the beginning and at the end of each experimental day. Moreover, experimental and lab controls run in parallel to reveal the influence of volume restriction, sealing and experiment conditions.

After irradiation, the embryos were separated in 96 well plates (Corning®, Merck) and maintained under normal conditions (28°C) including medium exchange every other day. Applying a Zeiss Axiovert S100 (25x magnification) embryonic survival and hatching were assessed daily, whereas morphological alterations, like pericardial edema (pe) and curved spines (sc), were recorded for the last two days of the four-day follow up. Both malformations were scored as binary event resulting in malformation rates correlated to the number of surviving embryos. At the 4^{th} day post-irradiation (dpi), pictures were taken from each surviving embryo before termination and fixation in 2 % paraformaldehyde (Sigma Aldrich) for further analysis. From these pictures, the embryo length and diameter of the eye were measured (ZEN, Version 2.6, Zeiss) and the severities of pericardial edema ($SV_{mean,PE}$) and spine curvature ($SV_{mean,SC}$) were assessed as described in [19]. Embryo length, eye diameter and severities were determined as mean values for the surviving embryos per sample. The survival was related to the number of living embryos at irradiation day.

Statistical analyses

One-way analysis of variance (ANOVA), followed by post-hoc pairwise comparisons corrected for multiple testing using the Tukey test, was applied to compare the endpoints between

reference irradiation and the UHDR pulse regimens as well as between the different pulse regimens for electron irradiation. For proton irradiation, the endpoints were compared between reference and UHDR by the two-sided t-test for independent samples. Correlations between the endpoints and the most important experimental parameters (dose, sealing and 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 the pulse regimen was independent of these experimental parameters, multivariable linear regression was additionally performed. Every endpoint that showed a significant result in the overall ANOVA test was individually considered as the dependent variable. Applied irradiation (one-hot encoded with reference irradiation as baseline) and the four experimental parameters were simultaneously included as independent variables. All analyses were performed with SPSS 27 (IBM Corporation, Armonk, NY, USA) and p-values below 0.05 were considered as statistically significant.

Results

Dose, time point of irradiation and, in a few cases, the experiment day were found to be significantly correlated to the experimental outcomes of electron irradiation (Table S2). However, the maximum difference in dose between the different electron regimes was only 0.7 Gy (Table S1), which rather indicates the quality of dose control during irradiation than a biological relevance at an irradiation dose of ~32 Gy. Irradiation time as surrogate for embryo age reflects the reduced radiosensitivity with a 10 % increase in embryo length comparing embryos irradiated at 6 pm (30 hpf) to those irradiated at 12 am (24 hpf, not shown) independent on the electron regime. This well-known effect [18, 21] cannot be circumvented completely at research accelerators with limited availability of beam time, but its influence on the outcome is minimized by irradiating one sample of each regime in each run resulting in equivalent ages. The significant effect of experiment day seen for PE, length and eye diameter could indicate a batch effect of different breeding pairs or some stress induced due to increasing workload from day to day during the experiment. In contrast to this observation, the

comparison of lab controls, pO_2 controls and experiment controls (Table S3) did not reveal significant differences indicating that volume restriction, oxygen consumption and the environmental conditions at the irradiation place did not alter embryo wellbeing and radiation response.

In total 35 – 36 samples were irradiated for each electron regime of which most of the embryos (>90 %) survived. A comparison of the mean values of all endpoints (Table 2) indicates that, except for survival and hatching rate, all UHDR regimes were beneficial for the embryos in comparison to reference irradiation. This was also confirmed by multivariable linear regression (Table S4) taking into account the above-mentioned dependencies on external parameters (dose, irradiation time and irradiation day). Exemplarily, the rates of pericardial edema are reduced by 7 – 12 % for $UHDR_{iso}$ and $UHDR_{synchro}$ and by 30 – 45 % for $UHDR_{max}$ summarizing the results at the 3rd and 4th dpi. The reduction in the rate of spinal curvature amounts to ~35 % for the $UHDR_{synchro}$ and $UHDR_{iso}$ regimes and to 52 % for irradiation at $UHDR_{max}$, respectively. The comparability of $UHDR_{synchro}$ and $UHDR_{iso}$ was confirmed by pairwise post-hoc tests (Table S5).

In order to detect underlying dependencies on beam pulse time structure, the biological outcome can be considered relative to the mean or bunch dose rates of the respective electron beam regimes. Exemplarily, the mean dose rate of the electron beam and the embryo body length (Figure 1a) are linearly correlated (R^2=0.98) showing higher protection, i.e. longer embryos, for higher mean dose rates. A similar linear dependency was revealed for the bunch dose rates (Figure 1b), except for the $UHDR_{synchro}$ regime that result in shorter embryos despite of a bunch dose rate of 10^9 Gy/s. Interestingly, the embryo length would fit quite well to the indicated linearity if the macro pulse dose rate (0.92×10^5 Gy/s) is considered as bunch dose rate.

The verification experiment at the UPTD cyclotron revealed a similar normal tissue protection of the high dose rate proton treatment (Table 3), compared to the quasi-continuous reference, as the $UHDR_{iso}$ electron regime (Figure 2). This finding of less damaging UHDR proton

irradiation was again confirmed by multivariable linear regression considering potential influences from external parameters, like dose, irradiation and sealing time, and experiment day (Table S6). Moreover, the proton beam parameter fit quite well to the observed mean dose rate dependency at ELBE (Figure 1a), whereas the bunch dose rates of 1.5×10^3 Gy/s for the UHDR proton regime and of 0.75 Gy/s for the proton reference irradiation, respectively, did not.

Discussion

The present study continues previous investigations on parameters that influence the radiation response of zebrafish embryos to UHDR radiation [18]. In the previous experiments at ELBE, the maximal inducible Flash effect and the impact of oxygen pressure at irradiation time were studied revealing a set of parameters circumscribing the requirements for inducing an electron Flash effect in zebrafish embryo [18]. Based on these observations, the present study focuses on beam pulse time structure using the ELBE electron beam to mimic the quasi-continuous beam delivery of a proton isochronous cyclotron ($UHDR_{iso}$) and the macro pulse structure of a proton synchrocyclotron ($UHDR_{synchro}$) [10]. These two UHDR regimes are characterized by comparable mean dose rates (Table 1, Figure S1) but differ in bunch dose rate by a factor of 1000, making the $UHDR_{synchro}$ regime comparable to the $UHDR_{max}$ in that particular point.

Despite differing beam pulse time structure, a protection of the zebrafish embryo from severe radiation damage was observed for all UHDR regimes and almost all endpoints (Table 2, Figure 1) relative to the reference regime. Here, the rates of pericardial edema and spinal curvature turned out to be the most sensitive endpoints with up to 45 % and 52 % reduction, respectively, comparing $UHDR_{max}$ and the reference regime. Focusing on the time structure of the beam, the time average mean dose rate was identified as the predominant factor that determines the extent of the Flash effect (Figure 1). The higher the mean dose rate or the shorter the treatment time, the less malformation were detected in the zebrafish embryo. This finding is strengthened by the comparability of $UHDR_{iso}$ and $UHDR_{synchro}$ for all endpoints, indicating that the common order of mean dose rate is more important than macro pulse

structure and bunch dose rate. In line with this, lower protecting effects were measured for $UHDR_{synchro}$ regime than for $UHDR_{max}$ despite of the same bunch dose rate (Figure 1b). These findings confirm the results of Ruan et al. [22] who observed a quartering in the number of surviving crypts by lowering the mean dose rate from 3.3×10^6 Gy/s to 280 Gy/s at constant high electron bunch dose rate of 10^6 Gy/s for whole abdomen irradiation of mice. Contrary to this finding, but also to the present results, no difference in embryo length was found after irradiation of 4 hpf zebrafish embryos with electron macro pulse dose rates of 10^6 Gy/s and 100 Gy/s [23], respectively. These contradictory results, but also others (review e.g. in [2, 7]), indicate that beside instantaneous (or bunch) dose rate the beam pulse time structure, i.e., macro pulsing, is relevant if the overall treatment time is prolonged. Exemplarily, for the delivery of 32 Gy in the present study 4000 electron bunches are used at $UHDR_{max}$ and $UHDR_{synchro}$ regime, but the macro pulse structure of the latter prolongs the treatment time from 300 µs to 168 ms and reduces the protecting Flash effect. A similar effect was observed when the treatment dose was split into two or five electron pulses for whole abdomen irradiation [22] with delivery time extension from 3.4 µs single pulse to 40 ms. Opposite observations are documented by [6, 23] with comparable results for one 1.8 µs electron pulse and 10 pulses delivered in ms range, respectively.

In line with the electron Flash effect seen for the $UHDR_{iso}$ regime, a similar reduction in radiation damage was found comparing 224 MeV proton beam delivery at 300 Gy/s UHDR and 0.15 Gy/s reference beam (Figure 2b), respectively. Contrary to our previous study, where no proton Flash effect was observed [14], setup and embryo handling were copied from the ELBE electron experiment. Instead of irradiation in 96 well plates without any oxygen control, the application of controlled, lower oxygen levels in Eppendorf tubes resulted in an observable Flash effect confirming the importance of oxygen pressure at irradiation time. Interestingly, the low bunch dose rate of the proton beam in the order of 10^3 Gy/s, compared to 10^6 Gy/s used for $UHDR_{iso}$, does not reduce the Flash effect confirming that mean dose rate and delivery time are the predominant factors. This corresponds to others studies, where a proton Flash effect

was observed at clinical isochronous cyclotrons of limited maximum bunch dose rate [4, 10, 13].

In conclusion, the present study demonstrates that the ELBE accelerator with its variable beam pulse time structure can be applied as a surrogate for different proton beams at least for LET values comparable with 0.274 keV/µm for 30 MeV electrons [24]. For the 224 MeV transmission protons (LET = 0.417 keV/µm), this fit quite well. For higher LET protons, studies at suitable proton accelerators are necessary to understand the influence of increased local energy deposition on the Flash effect [10, 15]. The zebrafish embryo model turned out to be an appropriate model for such studies helping to identify suitable beam parameters for more detailed experiments on the differential Flash effect, including normal and cancerous tissue in higher order organisms. Still open questions are, for example, if bunch dose rates higher than the 10^9 Gy/s applied in the present study will result in even higher protection and the impact of other parameters like treatment dose, dose fractionation and irradiation volume [7]. Whereas the latter could be answered in animal studies at clinical machines, alternative accelerator concepts are required to study the bunch dose rate dependency over a broad rate range of dose rates [4, 7, 25].

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Supplement

S1: Irradiation setup and dosimetry at the ELBE electron accelerator
S2: Irradiation setup at the experimental proton beamline at UPTD
S3: Dose delivery and absolute dose determination
Table S1: Mean dose values of the respective beam regimens
Table S2: Pearson correlation analysis to test the influence of different experimental parameters of the electron experiment on the respective endpoints.
Table S3: Mean lengths of control samples ± standard deviation (sd) for all experiments.
Table S4: Fit parameters and coefficient of determination (R) returned from the multivariable linear regression of different endpoints and the respective experimental parameters of the ELBE electron experiment.
Table S5: Comparison of electron regimens for those endpoints, where the overall ANOVA test was significant.
Table S6: Fit parameters and coefficient of determination (R) returned from the multivariable linear regression of different endpoints and the respective experimental parameters of the UPTD proton experiment.

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