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Original Article

Feasibility of proton FLASH effect tested by zebrafish embryo irradiation

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ABSTRACT

Background and purpose: Motivated by first animal trials showing the normal tissue protecting effect of electron and photon Flash irradiation, i.e. at mean dose rates of 100 Gy/s and higher, relative to conventional beam delivery over minutes the feasibility of proton Flash should be assessed.

Materials and methods: A setup and beam parameter settings for the treatment of zebrafish embryo with proton Flash and proton beams of conventional dose rate were established at the University Proton Therapy Dresden. Zebrafish embryos were treated with graded doses and the differential effect on embryonic survival and the induction of morphological malformations was followed for up to four days after irradiation.

Results: Beam parameters for the realization of proton Flash were set and tested with respect to controlled dose delivery to biological samples. Analyzing the dose dependent embryonic survival and the rate of spinal curvature as one type of developmental abnormality, no significant influence of proton dose rate was revealed. For the rate of pericardial edema as acute radiation effect, a significant difference ($p < 0.05$) between proton Flash and protons delivered at conventional dose rate of 5 Gy/min was observed for one dose point only.

Conclusion: The feasibility of Flash proton irradiation was successfully shown, whereas more experiments are required to confirm the presence or absence of a protecting effect and to figure out the limits and requirements for the Flash effect.

Introduction

During the last decades, radiotherapy dose delivery techniques were notably improved with respect to tumor conformity, e.g., by sophisticated beam delivery techniques like intensity modulated radiation therapy. One disadvantage also of modern photon beam techniques is the exposure of the surrounding normal tissue, which is linked to a risk of severe acute and late effects. Alternatives for reducing the risk are provided by the application of charged particles with their inverse depth dose profile or could be possible by the recently described Flash effect that promises normal tissue protection thanks to dose delivery by beam pulses of high dose rate [1]. Favaudon et al. [1] showed that electron Flash irradiated mice exhibit less lung fibrogenesis than mice treated with conventional electron beams whereas tumors react similarly to both treatment modalities. The better protection of the normal tissue was achieved by electron beam mean dose rates in the order of 100 Gy/s resulting in the delivery of doses up to 30 Gy within less than 500 ms [1]. Motivated by the first study, the protecting effect of electron Flash was meanwhile shown for cognitive defects after irradiation of mouse brains [2], severe radiation damage of mouse abdomen [3], the induction of skin damage in a mini-pig and the treatment of cat sarcoma [4]. Additionally, a recent study also confirmed the Flash effect for photon irradiation [5].

Patriarca et al. [6] published a first attempt for proton Flash irradiation applying a clinical cyclotron to deliver a proton spread-out Bragg peak with Flash-like parameters for future thorax irradiation of mice. In a similar manner, the present work describes the setup and parameters of proton Flash irradiation at a clinical proton machine as well as the first results obtained treating zebrafish embryos with proton Flash and conventional proton beam delivered at the University Proton Therapy Dresden (UPTD). In order to exclude potential LET (linear energy transfer) effects of low energy protons at the end of the proton path [7] irradiations were performed at the entrance plateau of the proton beam depth dose profile. Radiobiological effects of Flash versus conventional proton treatment were assessed by rating their influence on

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embryonic survival and the formation of morphological abnormalities [8].

Materials and methods

Beam setting and experimental setup

At the horizontal fixed-beam beamline in the experimental hall of UPTD [9,10] a setup (Fig. 1) was established that allows for the delivery of 224 MeV protons either by continuous beam at conventional dose rate of 5 Gy/min or by proton Flash (100 Gy/s). Due to the available beam current settings a polymethyl methacrylate scatterer (position 1) was deployed to reduce the proton fluence on central beam axes for the delivery of the conventional dose rate and to homogenize the dose distribution over the sample area of 6.5 mm in diameter. Similarly, a homogeneous dose distribution was achieved by placing the scatterer on position 2 with minimum reduction in the proton fluence for proton Flash. Thereby, dose homogeneity over the irradiation field was monitored with a Lynx scintillation detector (IBA Dosimetry GmbH, Schwarzenbruck, Germany) and by EBT3 dosimetry films (ISP Corp., New York, USA; supplement S1) at target position revealing maximum dose inhomogeneities of ±3%. The dose homogeneity along the proton depth dose curve was verified by Giraffe detector (IBA Dosimetry) measurements.

The dose delivery to the sample was monitored online by two ionization chambers (ICs): a beam monitor IC (BM-IC, model 34058 originally an OEM product of an intraoperative linear accelerator, PTW, Freiburg, Germany) at and a Bragg peak IC (BP-IC, model T34070-2,5, PTW) behind beam exit. Model 34058 is a segmented transmission IC, whereof the central segment was used for current readout of the proton beam. Both chambers were cross-calibrated against a capped Markus IC (M-IC, model 34045, PTW; cap: plastic, 1 mm water equivalent thickness) and EBT3 films at sample position. Recombination inside the ICs can be treated with the formalism of continuous irradiation [11], since the pulse duration (>100 ms) is much longer than the IC collection time (~10 μs). For the M-IC (electrode distance of 1 mm, applied voltage of 300 V) used for absolute dosimetry the recombination correction is according to the chamber data sheet 0.5% at a dose rate of 200 Gy/s and is therefore less and negligible for the mean dose rate of max. 100 Gy/s applied in this experiment. This was checked by measurements with the M-IC applying the two-voltage method. For the ICs used as monitor chambers the recombination correction is canceled by the daily determined correlation to M-IC for each dose rate regime. Furthermore, the actual dose was retrospectively determined by dose rate independent EBT3 films [12] calibrated in continuous proton beams (S2).

The dose was determined over the target area of 6.5 mm in diameter, which corresponds to the diameter of one well of a 96 well plate assuming the target area in the center of the beam spot. In order to estimate the dose uncertainty due to sample positioning uncertainty, the target area was randomly shifted laterally by 1 mm in x- and y-direction and the corresponding deviation in dose was determined for more than half of the films resulting in a positioning dependent dose uncertainty of ~1.0%. This leads, together with the dose inhomogeneity over the irradiated area, to a statistical accuracy of 4.5%. The systematic uncertainty of the absolute dose, which includes the uncertainties of calibration factors of the used ICs, temperature and air pressure corrections etc., don't need to be considered since the biological effects of both regimes were compared at same dose readouts and negligible recombination correction of M-IC.

Zebrafish embryo irradiation and follow up

The experimental protocol was planned according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and to the German legislation on the care and use of laboratory animals.

Wildtype AB Zebrafish embryos were kindly provided by the Center for Regenerative Therapies at Technische Universität Dresden. For irradiation, 21–22 hour post fertilization (hpf) embryos were washed and sorted into E3 medium [13], and transported to the proton facility with care of the necessary temperature maintenance. The embryos were kept at room temperatures (22–24 °C) until treatment in pharyngula stage (~24 hpf). Shortly before irradiation, 50 embryos were placed in one particular well of a 96 well plate, which was filled up with ~250 μl embryo medium and then enclosed with laboratory foil. To minimize the time in this confined geometry, irradiations were scheduled in runs of maximum six samples plus one non-irradiated control that was treated following the same procedure except irradiation. Owing to the fast switch both proton regimes were applied within each run avoiding any bias from irradiation sequence or time. Moreover, the samples were blinded to preclude observational bias. The influences of the maximum 1 h volume restriction during irradiation was checked by an additional non-irradiated control sample with about

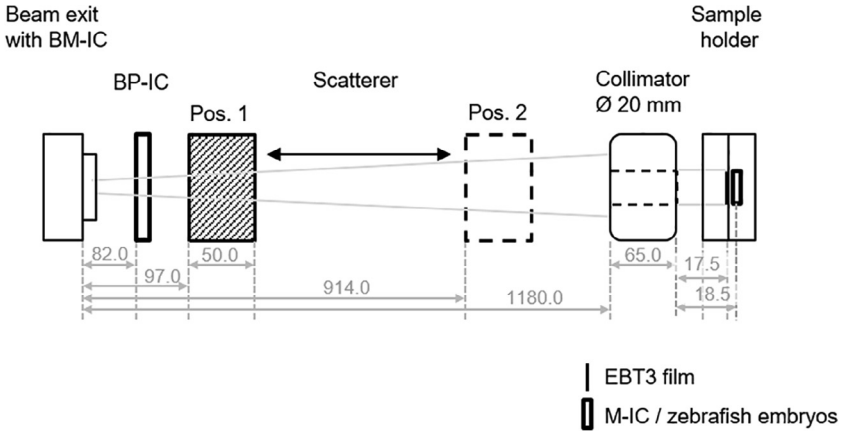

Fig. 1. Schematic representation of the experimental setup Coming from the left along the vacuum beamline the proton beam traverses the beam monitor chamber (BM-IC) at the beam exit into air, the Bragg peak chamber (BP-IC) as a second online dosimeter and the scatterer at position 1 for conventional proton beam or position 2 for proton Flash irradiation before being collimated to 20 mm diameter by a brass collimator. Finally, the proton beam enters the sample holder through an EBT3 film in front of either the Markus ionization chamber (M-IC) or the zebrafish embryos in 96 well plate. (Dimensions are in mm).

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50 embryos kept in one 80 mm petri dish in parallel to one experimental run. Following irradiation, the embryos were separated in one embryo per well and maintained under normal conditions (28 °C) including medium exchange (200 μl per well) every second day. Embryonic survival and morphological abnormalities (Fig. S1, Suppl. S2) were assessed daily applying similar observation periods for all samples and using a Zeiss Axiovert S100 at a magnification of 25× (Zeiss, Germany). At the 4th day post irradiation (dpi) the embryos were sacrificed and fixed in 2% paraformaldehyde for further histological analysis.

Experiment statistics

Three independent replications were performed for each dose group irradiated either with proton Flash or conventional proton beams. Survival rates were calculated relative to the number of treated embryos and average survival rates were determined as mean values of the three independent replications (see Supplement S2). The observed malformations were related to the number of surviving embryos and averaged over the three experimental sessions. Mean values of the three replicates were given with their respective standard deviation and Student's *t*-test was applied to compare iso-dose effects of both regimes assuming statistical significance for $p < 0.05$. The software Origin Lab 2017 (OriginLab Corporation, Northampton MA 01060, USA) was applied for graphical representation of average survival and malformation rates and for the determination of the ED50 (effective dose that produces an effect in 50% of embryos) and corresponding 95% confidence intervals by sigmoidal fit.

Results

The beam settings for both proton regimes (Table 1) together with the established online dose monitoring system were sufficient to deliver comparable doses to the zebrafish embryos as it was confirmed by retrospective absolute dose determination of the EBT3 films in front of each individual sample (Supplement S1).

The comparison of survival and malformation rates of the different control samples (Supplement S2) revealed that the embryos are quite robust to the confined space in a well and that no additional influence has to be taken into account. For example, mean survival rates of (98.5 ± 2.1) % and of (98.6 ± 0.8) % were obtained for the lab and the treatment control, respectively. The latter was applied as 0 Gy group for all samples independent on treatment regime.

The dose dependent embryonic survival data reveal a time dependent decrease for doses >15 Gy (Fig. 2, Suppl. 2), whereas no significant difference was observed with respect to proton dose rate. A similar finding was obtained for the time and dose dependent malformation rates, except for the rate of pericardial edema observed at the 3rd and 4th dpi after irradiation with 23 Gy (Fig. 2b). For that particular dose the rate of edema was significantly reduced after proton Flash irradiation ($p < 0.05$, Table S2). However, the overall dose response was not affected and comparable ED_{50} of 20 Gy [16.8; 22.7] and of 21 Gy [17.9; 24.5] were obtained at the 4 dpi with protons at 5 Gy/min and 100 Gy/s, respectively.

Table 1
Summary of proton beam parameters achieved for the two proton beam regimes in comparison to the parameters given for electron Flash.

	Conv. Proton beam	Proton Flash	Electron Flash
Position of scatterer	1	2	–
Current at beam exit/nA	0.3	95	–
Mean dose rate/Gy/s	0.08	100	>40 [1], >60 [2]
Macro pulses			
Frequency/Hz			≤200 [19]
Pulse length	–		~μs
Pulse dose rate/Gy/s	–		4.5×10^5 [19]
Micro pulses			
Frequency/GHz	0.106		5 [18]
Pulse duration/ns	2		n.a.
Pulse dose rate/Gy/s	0.4	0.5×10^3	3×10^7 [18]

Discussion

After first descriptions some decades ago [14,15], the tissue protecting effect of electron treatment at high pulse dose rate was rediscovered by Favaudon et al. [1] who observed normal tissue sparing under similar tumor killing efficacy. Subsequently, the protective Flash effect was successfully verified for MeV electrons [2–4] and 100 keV X-rays [5] and the technical feasibility of proton Flash irradiation has been proven [6]. Continuing this research, the present study addressed the required beam settings enabling the delivery of proton Flash with the clinical cyclotron of UPTD and the demonstration of its protective effect *in vivo*.

In general, a clinical system like the cyclotron Cyclone 230 installed at UPTD (Proteus® Plus clinical facility, IBA) is able to deliver Flash-like proton beams [6] with some limitations regarding maximum pulse dose and variability of pulse frequency and duration. Exploiting the available beam parameters, mean dose rates of 100 Gy/s and treatment times of less than 0.5 s comparable to those applied for electron Flash (Table 1) were achieved in the present experiment. However, a significant protective effect of proton Flash could be revealed neither for the survival nor for the morphological integrity of the zebrafish embryos. Solely for the rate of pericardial edema, a significantly reduced effect was found at the 3rd and 4th day after 23 Gy proton Flash compared to conventional proton irradiation.

The factors influencing the biological outcome of the experiment were mitigated as much as possible: a fast switch between proton beam regimes allows for high throughput experiments and random sample allocation. The influence of an increasing ionization density at the end of the proton track was minimized irradiating the samples at the entrance plateau of the depth dose distribution. However, although high-energy protons and electrons are both characterized as low LET radiations the spatial distribution of their energy deposition are clearly different at the microscopic scale and might affect the biological response.

From a biological point of view, the Flash effect needs to be demonstrated in a model that has predictive values for humans. However, the preceding experiment described in the present work makes use of zebrafish embryos at pharyngula stage for a first *in vivo* study of the proton Flash effect in general. Embryos at this stage are robust enough to resist volume-restriction during the experiment, but are concurrently less susceptible to radiation than embryos at earlier developmental stages [16,17]. For example, the electron Flash effect was recently observed for 4 hpf zebrafish embryos [17], whereas 24–28 hpf embryos were applied in the present study. Such a more sensitive model might of course be more appropriate to reveal a differential effect of proton dose rate, but the necessary time management from fertilization to experiment is very challenging. Alternatively, higher radiation doses could lead to more pronounced differences in the dose response curves, but the survival will be reduced to a level where the analysis of morphological malformations or histological endpoints is pointless.

Oriented by the beam parameters published for electron Flash [1–4] a mean dose rate of ~100 Gy/s and maximum irradiation

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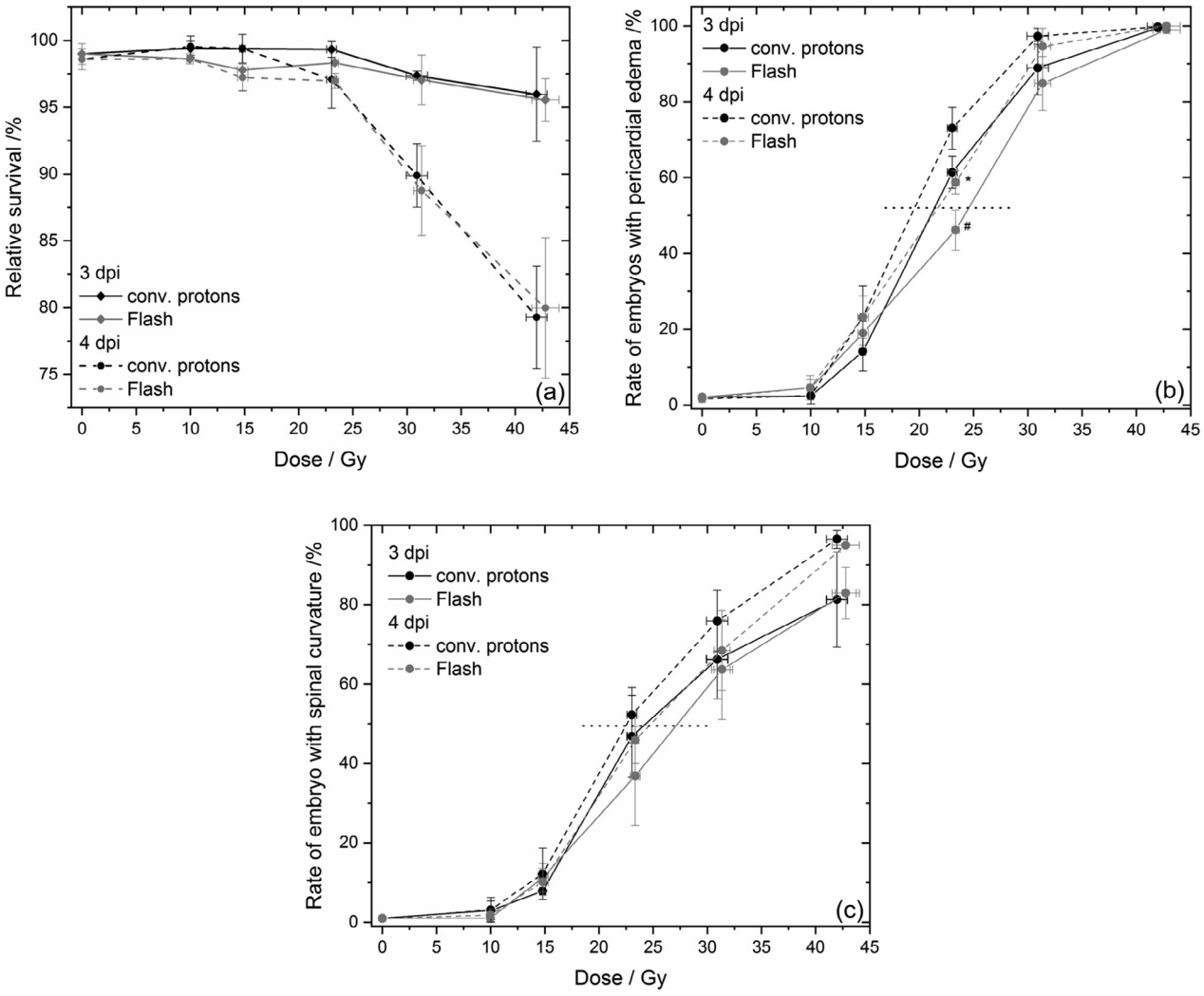

Fig. 2. Biological response to proton Flash and conventional proton beam irradiation Dose dependencies observed at the 3rd and 4th day post irradiation for the endpoints: (a) embryonic survival, (b) rate of pericardial edema and (c) rate of spinal curvature. Error bars represent the standard deviation of three independent experiment replications and dose uncertainty. Horizontal lines illustrate the doses required to induce 50% effect rate. Significant differences ($p < 0.05$) between both proton beam regimes were indicated by an asterisk for the 3 dpi and a hash key for the 4 dpi, respectively.

times of 400 ms were applied in the present work. Despite this similarity, a detailed look into the beam structure applied in the respective experiments reveal obvious differences in the macro and micro pulse parameters whose impact is not yet resolved but can a priori not be neglected. Table 1 summarizes the pulse parameters of the Kinetron [18] and Oriatron [19] linacs used for electron Flash and the UPTD cyclotron applied for proton Flash. Obviously, the quasi-continuous beam delivery of the cyclotron results in micro pulse dose rates of about 10^3 Gy/s several orders of magnitude lower than the 10^7 Gy/s estimated for the Kinetron [18]. The micro pulse structure of the two linacs is moreover superimposed by μs-long macro pulses with pulse dose rates of $\sim 10^6$ Gy/s [1,2] also exceeding the proton pulse dose rate. Whether these differences in beam pulsing are responsible for not observing a Flash effect in the present work should be investigated further also with the intention to clarify the parameters required to induce the protective effect of Flash irradiation. For this purpose, synchro-cyclotrons (e.g. S2C2, IBA) that deliver a pulsed beam with macro and micro pulse doses considerably higher than the quasi-continuous beams from isochronous cyclotrons such as cyclone 230 at UPTD [20] or research facilities like the ELBE electron accelerator [21] in Rossendorf could be applied. Particularly the latter allows variation of pulse dose and dose rate but also of pulse frequency and sequence over a very broad range enabling systematic studies on the physical parameters required for the Flash effect [22]. However, previous *in vitro* [23–25] and *in vivo* [26] studies with protons of even higher pulse dose rate resulted in inconsistent results showing either similar [23,24], reduced [25] or higher [26] biological efficiency relative to proton treatment over minutes.

Some decades ago, the protective effect of pulsed, high dose rate electron irradiation was primarily observed and linked to oxygen depletion in consequence of high dose impact in a very short time. Delivering a sufficient dose within one pulse, the consequential short-term hypoxia protects the irradiated cells [15] and tissue [14], whereas *in vitro* the effect was just seen irradiating under reduced oxygen level [27]. Current explanations of the protecting Flash effect also include the oxygenation status of the irradiated tissue favoring normal tissue instead of hypoxic tumors. However, the discussion on the oxygenation level, the actual oxygen consumption during irradiation and the kinetics of oxygen diffusion from well-oxygenated tissues around is ongoing. In addition to this, mechanisms like a differential DNA repair after pulsed and continuous irradiation [1], an altered immune response or

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something completely different are also conceivable. Focusing on the oxygen level, it is unclear whether zebrafish embryo that were maintained in an aquatic medium and take up oxygen by diffusion through their skin should be regarded as *in vitro* or *in vivo* sample and how their oxygen level could be defined. To clarify the influence of oxygen during Flash irradiation further experiments are required also with respect to the potential clinical application, where varying oxygen levels within the normal tissue and relative to the tumor are possible. However, quantitative measurements of the oxygen level, e.g., with an oxygen sensor, and systematic studies on its influence are unresolved. Such studies would be of interest not only for proton Flash but also for the general understanding and explanation of the Flash effect. Accordingly, forthcoming experiments should also include sophisticated models, like syngeneic or orthotopic tumor models that allow studying the Flash effect on normal and cancerous tissue simultaneously.

Declaration of Competing Interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.06.024.

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