Unraveling the proton FLASH effect

ABSTRACT

Background and purpose: Ultra-high dose rate (UHDR) irradiation has a protective effect on normal tissues compared to conventional (CONV) irradiation. However, the underlying biological mechanisms remain unclear. Here, we aimed to assess whether UHDR and CONV proton irradiation result in different levels of DNA damage in zebrafish embryos. Moreover, we studied the downstream transcriptional activation and functional changes following both modalities.

Material and methods: Zebrafish embryos, placed in closed tubes for 1h prior to irradiation, received 30 Gy UHDR (>5.1 kGy/s) or CONV (0.18 Gy/s) proton irradiation at 28h post-fertilization on a 68 MeV cyclotron. DNA damage was assessed by the alkaline comet assay at 4 hours post-irradiation. Gene expression changes were assessed at 6 and 24 hours post-irradiation using qRT-PCR including 20 radiation-responsive genes. Apoptosis, proliferation and neutrophil migration were investigated at 6 and 20 hours post-irradiation.

Results: Significantly higher levels of DNA damage were observed in CONV- versus UHDR-irradiated embryos. Moreover, CONV irradiation resulted in higher gene expression levels for most of the tested genes involved in DNA damage repair, apoptosis, ferroptosis and inflammation, with significant increases in *cdkn1a* and *mdm2* activity. Lastly, CONV irradiation resulted in higher levels of apoptosis in the embryonic tail compared to UHDR irradiation. Comparable proliferation and neutrophil migration to the sites of injury was found between the CONV and UHDR group.

Conclusion: UHDR proton irradiation induces less DNA damage and less downstream

transcriptional activation of DNA damage response pathways in zebrafish embryos compared to CONV irradiation, resulting in reduced embryonic cell death.

INTRODUCTION

Since the late 19th century, radiotherapy (RT) has been one of the main pillars of cancer treatment. Recently, ultra-high dose rate (UHDR) RT, also known as FLASH RT, has become one of the hottest topics in the radiation oncology field. Using dose rates exceeding 40 Gy/s, this irradiation modality has the capacity to reduce normal tissue toxicity while maintaining tumor control when compared to conventional dose rate (CONV) RT in preclinical setting, a phenomenon known as the FLASH effect [1-3]. Since the groundbreaking study published in 2014 [4], the number of scientific articles published on UHDR RT has been growing exponentially. Surprisingly, despite all the efforts, the biology behind the FLASH effect is not completely understood.

To date, most preclinical studies investigating the FLASH effect have been using mice – the gold-standard model in preclinical RT research. However, zebrafish (Danio rerio) embryos have emerged as a valuable alternative in vivo model in radiobiological (FLASH) studies. Upon exposure to ionizing radiation, specific well-described morphological abnormalities arise during embryonic development, including spinal curvature, body-length shortening and pericardial edema [5]. Zebrafish embryos are a particularly attractive in vivo model for mechanistic studies.

In our previous study, a protective effect of UHDR proton irradiation was found in terms of embryo body length and severity of pericardial edema compared to CONV irradiation [6]. Our results were consistent with previous observations reporting healthy tissue sparing in zebrafish embryos irradiated at different developmental stages using electron or proton UHDR RT [7-10].

However, the underlying biological mechanisms explaining this differential phenotype remain unclear.

Proton therapy is the most suitable technology for the clinical implementation of the FLASH phenomenon [11], thus we used proton irradiation in the current study. The aim of this study was to (1) assess radiation-induced DNA damage in zebrafish embryos following UHDR and CONV proton irradiation, to (2) examine transcriptional changes of the genes belonging to the canonical pathways activated by radiation exposure, and finally to (3) explore downstream functional events including apoptosis, proliferation, and immune cell migration following both treatment modalities.

MATERIALS AND METHODS

Zebrafish embryo handling and irradiation

Wildtype AB zebrafish embryos (Danio rerio) were kindly provided by XXXX in a portable incubator at 28°C where they were maintained in E3 medium at 28°C in a humidified incubator (Memmert IN75, Schabach, Germany). One hour prior to irradiation, 33 embryos in 100 µl of E3 medium were placed into closed 0.7 ml Eppendorf tubes containing polylactic acid (PLA) inserts. At 28 hpf, embryos were exposed to a proton dose of 30 Gy at either UHDR or CONV dose rates. Control embryos were also placed in closed Eppendorf tubes and sham-irradiated. Irradiation of zebrafish embryos was performed on four different days: batch 1, 2 and 3 were used on irradiation day 1, 2 and 3, respectively, and batch 4 and 5 were used on irradiation day 4. A schematic overview of the methodology is shown in Figure 1.

Alkaline comet assay

Single cell gel electrophoresis for detection of DNA damage was performed as described by Kosmehl and colleagues [12]. Zebrafish embryonic cells immobilized on microscopy slides were immersed in the lysis buffer at 4 h post-irradiation, ensuring fixation of the cells. More details are provided in Supplementary material. Two independent experiments were performed (batch 4 and 5), each with 3 samples of 21 embryos (derived from 3 different tubes) per condition.

RNA extraction

Embryos were fixed at 6 h and 24 h following irradiation by snap-freezing in liquid nitrogen. For RNA isolation, a combination of the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) extraction method and the clean-up on Qiagen RNeasy columns (Qiagen, Venlo, The Netherlands) was used. More details are provided in Supplementary material. Three independent experiments were performed for gene expression analysis (batch 1-3), each with 6 samples of 11 embryos (derived from 6 different tubes) per condition.

Reverse transcription and qRT-PCR

cDNA synthesis was performed using the RT2 First Strand Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. qRT-PCR was run using custom RT2 Profiler PCR Arrays and the RT² SYBR Green Mastermix (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). The list of genes present on the arrays is provided in Supplementary Table 1. qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method using dedicated software available at: http://www.qiagen.com/be/shop/genes-and-pathways/data-analysis-center-overview-page/custom-rt2-pcr-arrays-data-analysis-center/

Whole-mount immunofluorescence imaging

Embryos were euthanized and fixed in 4% formaldehyde at 6 h and 20 h post-irradiation and stored in 100% methanol at -20°C until further processing. Whole-mount immunostaining was performed as previously described [13]. More details are provided in Supplementary material. Images were recorded on a Zeiss LSM 880 confocal microscope. Two independent experiments were performed (batch 2 and 3), each with 8-12 embryos per condition (derived from 3 different tubes).

Viability and morphological abnormalities

Following irradiation, embryos were transferred into 96-well plates (one embryo/well) and incubated at 28°C up to 5 days post-fertilization (dpf) for assessment of viability, body length and spinal curvature. More details are provided in Supplementary material. Five independent experiments were performed (batch 1-5), each with 33-66 embryos per condition (derived from 3-6 tubes).

Statistical analysis

Normality analysis of data was completed with the Shapiro-Wilk normality test using Graphpad Prism (6.01) software. ANOVA tests were used to analyze the data that passed the normality test: one-way ANOVA for the body length comparison within each embryo batch and two-way ANOVA for gene expression analysis. Otherwise, data were analyzed by the Mann-Whitney test to obtain significant differences between the FLASH and CONV group. All data represent results from at least two independent experiments and are expressed as mean ± SD. Numbers of embryos used for analysis are indicated in the caption of the graphs. The p values < 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

RESULTS

UHDR proton irradiation spares zebrafish embryos from DNA damage

The alkaline comet assay was performed to investigate whether UHDR proton irradiation has the capacity to reduce DNA damage in zebrafish embryos compared to CONV proton irradiation. We found significantly lower levels of DNA fragmentation at four hours following UHDR vs. CONV irradiation (21.39 ± 5.12 % vs. 35.40 ± 7.02 % Tail DNA; $P < 0.01$) (Figure 2).

Reduced radiation-induced gene expression following UHDR proton irradiation

At 6 h post-irradiation, the expression of *cdkn1a*, a potent inhibitor of cell cycle progression in G1, was significantly increased in the CONV vs. UHDR group (1.25-fold increase in CONV vs. UHDR group; $P < 0.0001$) (Figure 3A). Moreover, expression of *ccnb1*, a key promotor of cell cycle progression from G2 to M phase, was downregulated after CONV PT, but not after UHDR PT (1.27-fold decrease in CONV vs UHDR; ns). At 24 h post-irradiation, a significant 1.22-fold increase after CONV irradiation (relative to UHDR irradiation; $P < 0.05$) was observed for *mdm2*, an important regulator of *p53* activity (Figure 3B). Moreover, a clear trend toward increased expression was found for the majority of the tested genes in CONV-irradiated embryos compared to UHDR-irradiated ones (1.32-fold increase on average after CONV vs. UHDR PT). The most pronounced differences were found for the immune response-related genes (*cxcl8a, tnfα, ifg1 and il6*). Additionally, the expression of the apoptosis-promoting genes (*casp3a, casp9 and baxa*) and the DNA damage repair genes (*mre11a, xrcc6, rad51 and pcna*) was higher in CONV- compared to UHDR-irradiated embryos, with *casp3a* (1.51-fold increase) and *xrcc6* (1.35-fold increase) being the most upregulated ones in these gene subgroups, respectively. Upregulation of the ferroptosis-promoting gene *acsl4a* was found in the CONV vs. UHDR group (1,27-fold increase in CONV vs UHDR).

UHDR proton irradiation results in less apoptosis in zebrafish embryos

We observed that, independent of the dose rate used, the most radiosensitive tissues in the embryos were the brain and eye neurons and the tail muscles. Therefore, we analyzed the apoptotic and proliferative behavior of embryonic cells in the tail and head region by cC3 and pH3 staining, respectively, at 6 and 20 h post-irradiation (Figure 4A). CONV PT resulted in significantly higher levels of apoptosis in the tail ($60.99 \times 10^3 \pm 23.79 \times 10^3$ vs. $46.25 \times 10^3 \pm 37.74 \times 10^3$ spots/μm^3; $P<0.05$) compared to UHDR PT at 20 h post-irradiation, which was concurrent with an increase in proliferation after CONV treatment. Higher apoptosis levels were also found in the head of CONV vs. UHDR-irradiated embryos, although the difference was not statistically significant. These data indicate that the reduction in radiation-induced DNA damage following UHDR PT may result in less embryonic cell death compared to CONV PT.

Neutrophil migration in zebrafish embryos is comparable after UHDR and CONV irradiation

To explore the possible immunomodulatory effects of UHDR proton irradiation on zebrafish embryos, migration of immune cells to the sites of injury was assessed at 20 h post-irradiation (Figure 5). We found that the percentage of MPO-positive neutrophils present in the head and the tail was higher for proton-irradiated embryos compared to non-irradiated controls. However, no significant differences were found between the CONV vs. UHDR groups in the head or outside the major blood vessels in the tail.

UHDR PT induces similar viability and morphological abnormalities as CONV PT

Viability of zebrafish embryos was increased following UHDR PT (87.44 ± 7.757 %) compared to CONV PT (82.75 ± 15.60 %), however, this increase was not statistically significant (Figure 6A). Next, similar body length measurements were found between the UHDR and the CONV groups (Figure 6B). In terms of curvature rate, no significant differences were observed between UHDR -and CONV-irradiated embryos (Figure 6C). Interestingly, one batch of embryos (batch 5)

showed a significant increase in viability rate (77.78 ± 9.623 % vs 55.56 ± 9.622 %; $P < 0.05$) and body length (3164.79 ± 256 µm vs 3016.95 ± 129.4 µm; $P < 0.05$) after UHDR vs CONV treatment (Figure 6D-E).

DISCUSSION

In recent years, the normal tissue sparing effects of UHDR irradiation have astonished many researchers in the radiation oncology field. However, the underlying mechanisms of action have not been clarified yet. On the biological level, the FLASH sparing effect on normal tissues can be the consequence of reduced DNA damage, less inflammation, sparing of circulating immune cells, normal stem cell sparing and vasculature preservation [14-16].

Previously, we demonstrated that UHDR proton radiation reduced developmental abnormalities such as body length shortening and pericardial edema in 5 dpf zebrafish embryos compared to CONV proton radiation [6]. To get a deeper insight into the upstream processes which eventually give rise to these morphological changes, we took advantage of the mechanistic potential of the zebrafish embryo model. The suitability of zebrafish embryos as a model of healthy tissue toxicity in the context of UHDR RT studies has already been demonstrated by several groups [7-10, 17]. In the current study we investigated DNA damage, transcriptional changes and downstream functional processes affected by radiation exposure in 28 hpf zebrafish embryos exposed to 30 Gy of CONV and UHDR proton irradiation.

First, we explored the potential of UHDR proton irradiation to reduce DNA damage in zebrafish embryos compared to CONV irradiation by means of the alkaline comet assay. A significant reduction in total DNA damage formation in the embryonic cells was shown at four hours following UHDR vs. CONV irradiation. Our results are in close agreement with previously reported in vitro [18-20] and in vivo [21] data. Ohsawa et al. demonstrated that UHDR proton

irradiation reduced the number of DNA single-strand breaks and not double-strand breaks (DSBs) in aqueous plasmid DNA compared to CONV proton irradiation [22]. This suggested that the observed increase in DNA damage in the CONV group may not necessarily result in lethal DNA damage

We also investigated the impact of UHDR irradiation on the expression of genes belonging to the canonical pathways involved in the response to ionizing radiation. A selection of genes was made based on preliminary experiments and literature studies [23-26]. We found that the expression levels of *cdkn1a* and *mdm2* were significantly reduced in UHDR vs. CONV-irradiated embryos at 6 and 24 hours post-irradiation, respectively. *Mdm2* is a ubiquitin ligase stimulating proteasomal degradation of *p53* under normal conditions [23]. However, DNA damage causes *p53* phosphorylation and dissociation from *mdm2*, resulting in *p53* accumulation in the nucleus where it induces transcription of several DNA damage response (DDR) genes including *mdm2* (negative-feedback loop) and *cdkn1a*, a potent inhibitor of cell cycle progression in G1 [27]. Moreover, a clear trend toward lower expression levels for the majority of the tested genes involved in several DDR-related pathways (DNA damage repair, apoptosis, ferroptosis, and inflammation) was found after UHDR vs. CONV treatment. The involvement of lipid peroxidation in the FLASH effect was also recently explored by Froidevaux et al. who demonstrated that lipid peroxidation, driving ferroptosis, was enhanced in liposomes after CONV irradiation but not after UHDR irradiation [28]. Interestingly, we also observed higher upregulation of the ferroptosis-promoting gene *acsl4a* in the CONV vs. UHDR group in our zebrafish embryo model.

To get more insight into the downstream cellular events, we explored the apoptotic and proliferative behavior of cells from UHDR and CONV-irradiated embryos by performing cC3 and

pH3 immunostaining, respectively. At 20 hours post-irradiation, we found significantly higher levels of apoptosis in the tail of CONV vs. UHDR-irradiated embryos. These results are in line with several in vivo studies reporting less apoptosis in mice following UHDR vs. CONV irradiation [4, 21, 29]. No major differences in the number of pH3-positive cells were found between the UHDR and CONV group. An immunomodulatory effect has also been suggested to (partially) explain the normal tissue sparing by UHDR irradiation. In our previous study, CONV irradiation induced more severe pericardial edema [6], a finding also reported by other researchers [7, 8, 17]. This study found higher induction of immune response-related genes in the CONV group. We investigated the inflammatory response functionally, quantifying neutrophils in the head and tail of embryos, as they are rapidly recruited to sites of radiation-induced damage [30, 31]. However, at 20 hours post-irradiation, similar neutrophil numbers were observed in both treatment groups.

Viability and morphological abnormalities (body length-shortening and spinal curvature) were found similar between UHDR and CONV-irradiated embryos. This is in contrast to our previously reported results which showed a clear FLASH sparing effect in terms of body length. In this study we also found a significant increase in viability rate and body length in UHDR vs. CONV-irradiated embryos from batch 5, which was not the case for embryos from the other batches (batch 1-4). The reason for this discrepancy may be dual. First, there might be a natural variation in robustness and radiosensitivity between the embryo batches. Second, the effect of initial oxygen concentration on FLASH sparing was highlighted in the study by Pawelke et al. in which CONV-irradiated embryos at low oxygen concentration had body lengths that were comparable to those from UHDR-irradiated embryos at high oxygen concentration [7]. For our oxygen measurements in the Eppendorf tubes, the oxygen probe was positioned with the tip approximately 0.5 mm above the PLA, resulting in a consistent oxygen concentration drop to

0.5% at 30 min incubation time (Supplementary Figure 1). However, it is not practically feasible to monitor oxygen concentration in every tube nor at every point within the tube during the irradiation, thus the possible variation in oxygen concentration within and between the tubes might explain the observed differences.

In summary, our results show for the first time that increased DNA damage caused by CONV vs. UHDR proton irradiation in zebrafish embryos is linked to the transcriptional activity of multiple pathways, notably in cell cycle arrest and p53 regulation. Additionally, UHDR proton irradiation significantly reduces apoptosis induction in the embryonic tail. Further research should confirm whether the magnitude of differences in DNA damage and downstream processes observed in a low vertebrate animal model is large enough to translate into relevant sparing effects in the clinic.

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Figure 1. Experimental design of the study

Irradiation of zebrafish embryos was performed on four different days: batch 1, 2 and 3 were used on irradiation day 1, 2 and 3, respectively, batch 4 and 5 were used on irradiation day 4. Following 30 Gy proton irradiation, embryos were euthanized and fixed at different timepoints to test several endpoints. *Hpi: hours post-irradiation; dpi: days post-irradiation; hpf: hours post-fertilization; PLA: polylactic acid; cC3: cleaved Caspase-3; pH3: phosphorylated histone H3; MPO: myeloperoxidase.*

Figure 2: UHDR PT causes less DNA damage in zebrafish embryos

(A) Alkaline comet assay measures of DNA damage formation (% Tail DNA) in cells of zebrafish embryos at 4 h following 30 Gy UHDR or CONV PT. The graph shows the data of two independent experiments, each with 3 samples of 21 embryo (derived from 3 different tubes) per condition; error bars indicate SD. A Mann-Whitney test was performed to test statistical significance (**, $p<0.01$). (B) Representative alkaline comet assay images as revealed by SYBR Green staining of the cells of non-irradiated control embryos and 30 Gy UHDR- and CONV-irradiated embryos.

Figure 3. UHDR PT decreases the induction of radiation-responsive gene expression

Relative expression of 20 genes from zebrafish embryos exposed to 30 Gy UHDR or CONV PT and fixed at (A) 6h and (B) 24h post-PT was measured by real-time qPCR. The graphs show the data of three independent experiments, each with 6 samples of 11 embryos (derived from 6 different tubes) per condition; error bars indicate SD. A two-way ANOVA test was performed (*, $p<0.05$; ****, $p<0.0001$).

Figure 4. Apoptosis and proliferation in head and tail region of zebrafish embryos after UHDR and CONV PT

(A) Quantification of apoptosis and proliferation in the head and tail region of zebrafish embryos measured by cC3 and pH3 staining, respectively, at 6 and 20 h following 30 Gy UHDR or CONV PT. Results are the average of duplicate experiments, each with 8-12 embryos per condition; error bars indicate SD. Representative images of cC3 -and pH3-positive cells in (B) the head and (C) the tail of zebrafish embryos at 20 h post-irradiation.

Figure 5. Immune cell migration to the head and tail region in zebrafish embryos after UHDR and CONV PT

(A) Quantification of immune cell migration to the head and outside the vasculature in the tail of zebrafish embryos measured by MPO staining at 20 h following 30 Gy UHDR or CONV PT. Results are the average of duplicate experiments, each with 8-12 embryos per condition; error bars indicate SD. (B) Representative images of the distribution of MPO-positive cells in zebrafish embryos at 20 h post-irradiation.

Figure 6. Viability and morphological abnormalities after UHDR and CONV proton irradiation.

Viability rate, body length and curvature rate of zebrafish embryos was assessed at 4 days following 30 Gy UHDR and CONV irradiation. Panel A-C: data derived from five independent experiments (embryo batch 1-5), each with 33-66 embryos (3-6 tubes) per condition; error bars indicate SD. Panel D-E: data derived from one experiment (embryo batch 5), with 33 embryos (3 tubes) per condition; error bars indicate SD. Symbols represent (D) the viability rate of embryos per tube, (E) the body length of a single embryo and (F) the curvature rate of embryos per tube. *, $p<0.05$.