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PHYSICS CONTRIBUTION

Proton FLASH: Impact of Dose Rate and Split Dose on Acute Skin Toxicity in a Murine Model

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Purpose: Preclinical studies have shown a preferential normal tissue sparing effect of FLASH radiation therapy with ultra-high dose rates. The aim of the present study was to use a murine model of acute skin toxicity to investigate the biologic effect of varying dose rates, time structure, and introducing pauses in the dose delivery.

Methods and Materials: The right hind limbs of nonanaesthetized mice were irradiated in the entrance plateau of a pencil beam scanning proton beam with 39.3 Gy. Experiment 1 was with varying field dose rates (0.7-80 Gy/s) without repainting, experiment 2 was with varying field dose rates (0.37-80 Gy/s) with repainting, and in experiment 3, the dose was split into 2, 3, 4, or 6 identical deliveries with 2-minute pauses. In total, 320 mice were included, with 6 to 25 mice per group. The endpoints were skin toxicity of different levels up to 25 days after irradiation.

Results: The dose rate$_{50}$, which is the dose rate to induce a response in 50% of the animals, depended on the level of skin toxicity, with the higher toxicity levels displaying a FLASH effect at 0.7-2 Gy/s. Repainting resulted in higher toxicity for the same field dose rate. Splitting the dose into 2 deliveries reduced the FLASH effect, and for 3 or more deliveries, the FLASH effect was almost abolished for lower grades of toxicity.

Conclusions: The dose rate that induced a FLASH effect varied for different skin toxicity levels, which are characterized by a differing degree of sensitivity to radiation dosage. Conclusions on a threshold for the dose rate needed to obtain a FLASH effect can therefore be influenced by the dose sensitivity of the used endpoint. Splitting the total dose into more deliveries compromised the FLASH effect. This can have an impact for fractionation as well as for regions where 2 or more FLASH fields overlap within the same treatment session.

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Data Sharing Statement: Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

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Introduction

A key challenge in radiation therapy (RT) for cancer is to maximize the radiation effect in cancer cells while minimizing damage to surrounding healthy tissue and thereby increasing the therapeutic ratio: the balance between cure and toxicity of treatment. One of the most promising and debated new RT modalities is FLASH therapy, where the radiation dose is delivered with an ultra-high dose rate around 1000 times higher than conventional dose rates of ~0.05 Gy/s. FLASH was suggested in 2014 by Favaudon et al, when electron beam experiments surprisingly demonstrated normal tissue sparing effects while an unchanged effect on tumor response[1-3] was observed.

However, the factors influencing the induction and magnitude of the observed FLASH sparing effect in normal tissue are not well understood. From the current literature, it has been demonstrated that dose rate and overall treatment times are crucial parameters to elicit a FLASH effect.[4-6] The definition of the optimal dose rate required to achieve the FLASH sparing effect remains an intense subject of research, and it is apparent that other factors, such as time structure of the total dose delivery, may also affect the FLASH effect.[7] For proton pencil beam scanning (PBS), there is yet no consensus on the most relevant definition of the dose rate. The field dose rate, ie, the total dose delivered divided by the total field duration, is a simple and conservative metric, but it does not account for the significantly varying local dose rate.[8] So far, comparison of dose rate definitions with radiobiological outcome data have not been performed, but these are necessary to determine which one reflects the radiobiological response the best. This is needed both to enable data comparison between experiments and for the approaching proton FLASH treatment plan optimization.

In previous studies, we have demonstrated a normal tissue sparing effect of proton FLASH for both acute and late damage in mouse tissue, while tumor control was unaffected by the increased dose rate. This was done through full dose response curves, which enabled quantification of the tissue sparing factor, the so-called FLASH factor, and revealed that although both acute and late effects were decreased by using FLASH irradiation, the late effects seemed to be less spared than the acute effects.[9,10] These studies were performed using PBS and compared the lowest obtainable field dose rate with the highest possible field dose rate within our system. Keeping the dose rate as low as possible was done by, among other methods, delivering the field with repainting, meaning that the field was painted 189 to 320 times with 4.3 ms pauses between each spot delivery to reduce the field dose rate.

The current study is using the same model and setup to investigate how the time structure of the beam delivery influences the FLASH effect. This is done through testing varying dose rates to obtain dose rate response curves and by comparing the influence of repainting on the FLASH effect. Furthermore, the effect of splitting the dose into smaller deliveries was evaluated, simulating the effect of delivering multiple overlapping fields.

Methods and Materials

Mouse setup and response

Experiments were performed on 16- to 20-week-old female CDF1 mice, with the different ages distributed between the treatment groups. Animals were acquired from Janvier Labs (France) and transported together under conditions that minimize stress during travel. Before treatment, the mice were acclimatized for 4 weeks in a housing of 3 to 4 mice per cage. The animals were randomly divided into cages. Only females were used in the experiments to circumvent any sex differences. The cage temperature was 22°C to 23°C with a dark and light cycle of 12 h. Ad libitum food and water were accessible, and the mice were weighed every seventh day during the follow-up period. The number of mice per treatment group was initially planned based on data from previous dose-response studies using FLASH dose rates in this murine skin toxicity model.[9]

The procedure for radiation treatment has previously been described.[9,11] Irradiations were performed locally to the right leg of nonanesthetized mice restrained in a Lucite jig. The legs were attached to a jig with glue. The mouse jigs were placed on a lucite plate fixed on the top of a water bath, with the target leg placed into the water bath. The temperature of the water bath was kept at 25°C (23.9°C-25.9°C). All mice were treated in the same time interval of the day (16:00-23:00).

The skin toxicity was assessed in the irradiated mice using a previously published skin score table[12] as described previously.[9,13] The skin damage in an area covering the whole foot was evaluated and scored in steps of 0.5, with 3.5 being the maximum value on a daily basis between 11 and 25 days after treatment.

Mice were irradiated in 3 separate experiments (Table 1), executed in 6 separate experimental runs over a period of 14 months. A total of 320 mice were included in the final analyses. All scorings and evaluations of the animals during follow-up were performed blinded to the given treatment. All experiments complied with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and were performed under the Institutional and National Guidelines for Animal Welfare. The study was approved by the local experimental institutional review board.

Dose delivery

All treatments were performed with proton PBS at the fixed horizontal proton beam line at the Danish Centre for Particle Therapy at Aarhus University Hospital (ProBeam, Varian Medical Systems, Palo Alto, CA, USA). The mouse setup and treatment fields have been described previously[9,10] and will only be summarized briefly here. The irradiated mouse leg was placed in the entrance plateau of the depth dose curve in a treatment depth of 13.5 cm with the mouse body shielded from direct radiation by a brass

Table 1 Parameters for animals treated in the three individual experiments

Planned field DR (Gy/s)	Actual field DR (Std) (Gy/s)	Beam duration (Std) (s)	PBS DR (Std) (Gy/s)	Overall treatment duration (Std) (s)	Number of Mice per group	Requested beam current (nA)	Number of repaintings	Number of deliveries	Dose pr delivery (Gy)	Alanine measurement (Gy) (Std)
Experiment 1: Varying dose rate - without repainting										
0.7	0.66 (0.09)	60.5 (6.9)	1.73 (0.24)	60.5 (6.9)	7	1.6-2.07	1	1	39.3	37.4 (0.6)
2	1.97 (0.10)	20.0 (1.0)	5.17 (0.25)	20.0 (1.0)	6	5.2-5.3	1	1	39.3	37.5 (0.7)
5.5	5.6 (0.4)	7.10 (0.51)	14.6 (1.1)	7.10 (0.51)	16	14.5-17.7	1	1	39.3	38.7 (0.7)
20	19.3 (1.0)	2.04 (0.11)	50.8 (2.7)	2.04 (0.11)	18	55.6-57.5	1	1	39.3	39.3 (0.6)
40	39.4 (1.3)	1.00 (0.03)	103.5 (3.4)	1.00 (0.03)	18	95.3-123.8	1	1	39.3	39.2 (0.6)
60	58.7 (1.5)	0.67 (0.02)	154.4 (3.9)	0.67 (0.02)	19	141.3-183	1	1	39.3	39.0 (1.0)
80	79.3 (9.2)	0.50 (0.06)	208.6 (24.1)	0.50 (0.06)	25	215	1	1	39.3	39.0 (0.9)
Experiment 2: Varying dose rate - with repainting										
0.37	0.37 (0.01)	107.8 (3.7)	0.37 (0.01)	107.8 (3.7)	13		320	1	39.3	38.5 (1.1)
0.7	0.67 (0.10)	59.9 (7.5)	0.66 (0.10)	59.9 (7.5)	8	1.2-2.07	31	1	39.3	37.4 (0.5)
2	1.93 (1.13)	20.5 (1.4)	1.90 (0.13)	20.5 (1.4)	7	5.2-5.3	31	1	39.3	37.7 (0.5)
5.5	5.4 (0.4)	7.37 (0.66)	5.3 (0.4)	7.37 (0.66)	15	14.5-17.7	31	1	39.3	38.5 (0.4)
20	19.8 (0.9)	1.98 (0.09)	20.0 (0.9)	1.98 (0.09)	10	55.6-56.1	18	1	39.3	39.1 (0.6)
40	38.6 (0.7)	1.02 (0.02)	40.9 (0.8)	1.02 (0.02)	9	95.3-115.6	9	1	39.3	39.1 (0.7)
60	59.4 (2.0)	0.66 (0.02)	64.4 (2.1)	0.66 (0.02)	11	141.3-183	6	1	39.3	38.6 (1.1)
80	88.8 (3.6)	0.44 (0.02)	102.9 (4.1)	0.44 (0.02)	13	215	4	1	39.3	38.5 (0.4)
Experiment 3: Split dose										
0.37	0.36 (0.02)	108.2 (5.9)	0.36 (0.02)	108.2 (5.9)	17		320	1	39.3	39.9 (0.2)
0.37	0.35 (0.02)	111.7 (4.9)	0.35 (0.02)	892.3 (48.6)	13		320	6	6.6	39.6 (0.4)
60	57.7 (1.5)	0.68 (0.02)	151.7 (4.0)	0.68 (0.02)	19	174.1-190.6	1	1	39.3	39.9 (0.2)
60	59.5 (2.9)	0.66 (0.03)	156.4 (7.7)	122.5 (2.1)	19	176.7-194.1	1	2	19.7	39.9 (0.4)
60	59.8 (4.0)	0.66 (0.04)	157.1 (10.5)	242.8 (2.1)	18	174.1-194.1	1	3	13.1	39.9 (0.4)
60	60.9 (4.9)	0.65 (0.05)	159.9 (12.9)	379.2 (33.1)	18	176.7-190.6	1	4	9.8	40.0 (0.5)
60	60.0 (2.5)	0.66 (0.03)	157.6 (6.7)	607.3 (9.2)	21	174.1-190.5	1	6	6.6	40.1 (0.4)

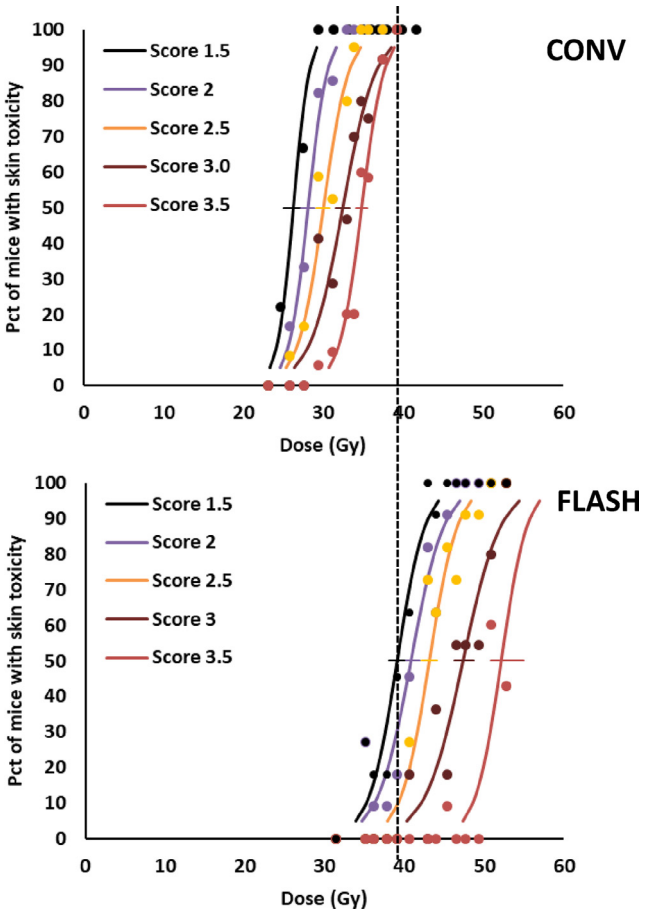


Fig. 1. Dose dependency of acute skin toxicity. Dose response curves of 5 levels of acute damage to the skin at either the conventional dose rate or FLASH dose rate. Skin reaction was scored categorically, where scores of 1.5 and 2.0 represent milder skin reaction; 2.5, a medium skin reaction; and 3.0 and 3.5, severe skin toxicity. The 95% confidence intervals are indicated at the MDD50 (dose causing skin toxicity in 50% of mice). Data are from reference 9. The used dose of 39.3 Gy is indicated with a dashed line.

block. Each mouse was irradiated with a 2 × 3-cm field that consisted of 5 × 7 spots with 5-mm vertical separation and 5.1-mm horizontal separation in the isocenter plane.

The mice were treated with a total prescribed physical dose of 39.3 Gy. Parameters for the individual experiments can be seen in Table 1. The selected dose was based on previous dose-response studies using FLASH (80 Gy/s, delivered without repainting) and conventional (CONV, 0.37 Gy/s, delivered with repainting) dose rates in this murine skin toxicity model.[9] Here, the 39.3 Gy dose gave a large separation between the CONV and FLASH dose rates, with severe skin toxicity observed in all mice that received CONV irradiation, while FLASH only gave a small risk of mild toxicity (Fig. 1).

In experiments 1 and 2, different dose rates were used. The dose was delivered either without repainting (Experiment 1) or with repainting (Experiment 2). The field dose rate of 0.37 Gy/s was only used in experiment 2, with a beam energy of 244 MeV and 320 repaintings. The higher field dose rates were delivered using 250 MeV and 4 to 31 repaintings. The number of repaintings decreased with increasing field dose rates and was chosen as the highest possible number that fulfilled the technical requirements of at least 100 monitor units and a 3-ms duration per spot (Table 1). The effect of repainting on the time structure of the beam delivery can be seen in Figure 2, where an example of the instantaneous dose rate and the cumulative dose in a central point of the field can be seen. The effective time interval for delivering most of the dose in a point was nearly the total field duration with repainting, whereas it was considerably shorter without repainting. For a given field dose rate, the 2 treatment groups with and without repainting had the same field duration and distribution of instantaneous dose rates, but with repainting, the dose delivery to a given point was spread more evenly over the entire field duration (Fig. 2D).

In Experiment 3, the 39.3-Gy dose was delivered with a fixed field dose rate of 0.37 Gy/s with repainting or 60 Gy/s without repainting. The dose was divided into 1 to 6 equal deliveries with pauses in between. Three mice were treated at a time with individual fields, and the pauses were aimed at 120 s between each round of 3 mice. For CONV, that gave somewhat longer mean pauses of 156.1 s (±9.8 s), because each pause included treatment of the other 2 mice in the round. For FLASH, the treatment time for the other 2 mice in the round was close to negligible, and the mean duration of the pauses was 121 to 126 s.

For all irradiations with field dose rates higher than 0.37 Gy/s, the field was delivered using 250 MeV and a variable requested beam current in research mode. A prototype integrated ultra-high dose rate beam monitoring and control system ensured correct dose delivery at all applied dose rates. For field dose rates of 0.7 to 60 Gy/s, a test field was delivered each day and before each change of dose rate, using an initial requested beam current. The field dose rate of the test delivery was determined as the dose divided by the logged field duration, as previously validated,[14] and the requested beam current was adjusted to give the field dose rate of interest. For field dose rates of 80 Gy/s, the highest possible beam current, 215 nA, was used. During the period when the individual experiments were conducted, a technical update of the beam delivery system was performed, and thereafter it was not technically possible to deliver the beam with the low dose rates (≤2 Gy/s) at 250 MeV. The number of included mice in these treatment groups was therefore restricted.

The absolute dosimetry was based on measurements with an Advanced Markus chamber as described previously.[10] The radiation dose contamination in a mouse leg from irradiation of neighbor mice in the water bath was previously measured at 1.0% to 1.5% per neighboring mouse.[10,15] This dose contribution was neglected in this study.

For all irradiations, the duration of each spot delivery was extracted from machine log files.[14] The field dose rate was

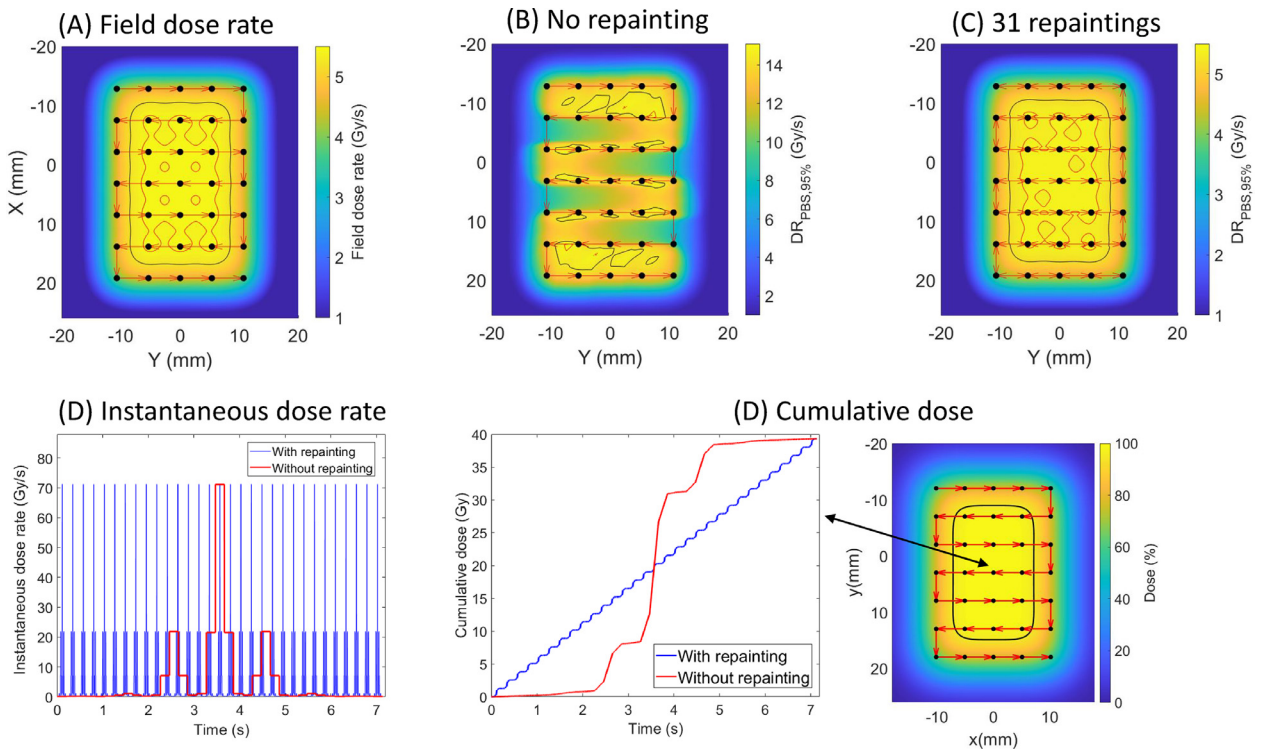


Fig. 2. Time structure of dose delivery with and without repainting. Field dose rate (A) and 95% pencil beam scanning dose rate (B) without repainting and (C) with 31 repaintings for a 5.5 Gy/s field delivery. The red and black contours show the 99% and 95% dose rate levels, with 100% being the maximum dose rate. Instantaneous dose rate (D) and cumulative dose as a function of time in the central point of the spot pattern without repainting (red) and with 31 repaintings (blue) (E, left). The proton field consisting of 7 rows with 5 spots per row (E, right).

determined as the total dose divided by the total field duration. The duration of the beam pauses between spots for CONV was not directly available in the machine log files. Based on previous measurements, it was assumed to be 4.3 ms. The local PBS dose rate for the 95% dose ($DR_{PBS95\%}$) was defined as 95% of the dose at a point divided by the time interval between reaching 2.5% and 97.5% of the dose at the point.[8] The $DR_{PBS95\%}$ was calculated in the middle of the field (Table 1).

As previously described,[10] alanine in vivo dosimetry was performed for individual mice using an alanine pellet placed in the middle of the field at the beam entrance on the water bath (Table 1). In experiments 1 and 2, alanine measurements were available for all mice, whereas in experiment 3, there were alanine measurements for 114 of 140 mice. In experiment 3, the alanine dosimetry indicated that all 15 mice treated on 1 treatment day had received a 10% higher dose. As no explanation could be found for this higher dose measurement, these mice were excluded from the final data.

Data analysis

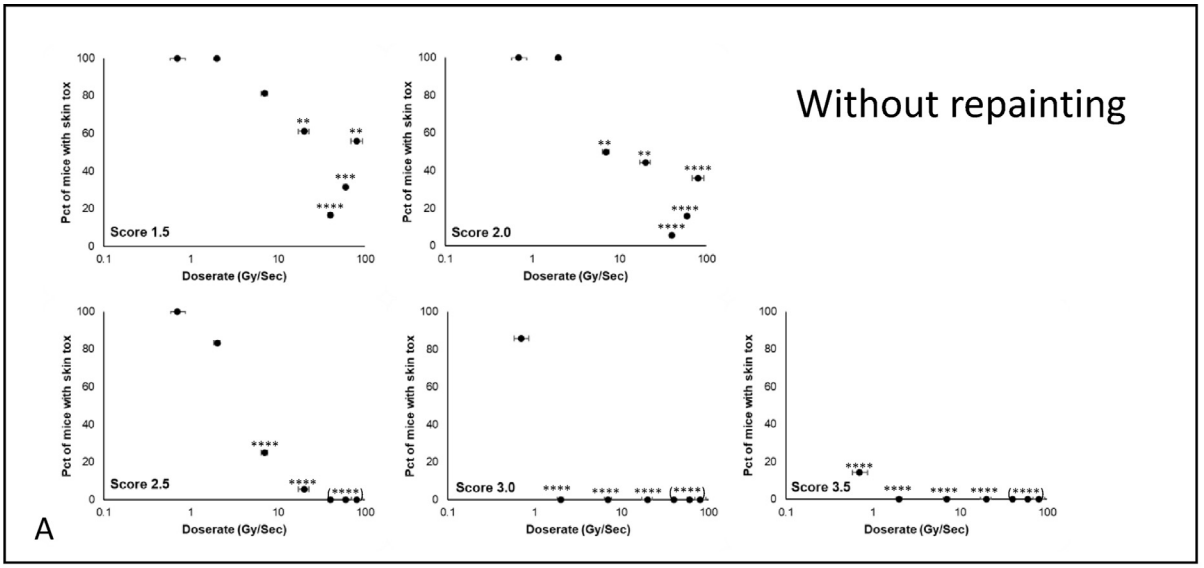
For data analysis and graphical illustration of the biologic data, GraphPad Prism 9.4 and Rstudio were used. To determine the radiation response of skin toxicity, the percentage of animals in each group with a certain score level (1.5, 2.0, 2.5, 3.0, or 3.5) at least once at any time during follow-up was calculated. This procedure is robust to differences in the time dependency of the maximum score reached in the different treatments. The individual data in the different scores are dependent between scores, as to reach a score 3.0, a mouse must also have had scores of 1.5, 2.0, and 2.5.

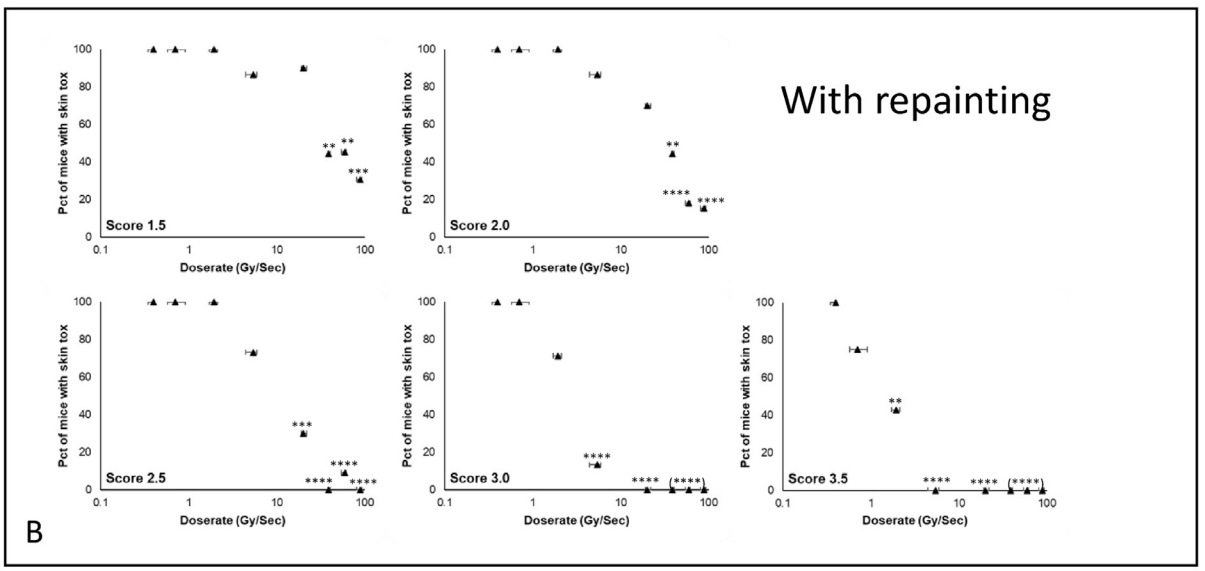
Statistical significance between treatment groups was calculated with Fisher's exact test.

Skin reactions as a function of time after radiation in the different treatment groups were calculated as the median skin reaction score in the group on the specified day. To allow for an estimation of a dose $rate_{50}$ value (the dose rate where 50% of the animals display toxicity), a piece-wise linear relationship on the gradient part of the curves was assumed.

Results

In a previous study,[9] the effects of CONV and FLASH were evaluated by comparing the lowest obtainable dose rate with the highest possible dose rate obtained at the maximum requested nozzle current of 215 nA, which led to large dose rate variations between treatment days. When comparing the different toxicity levels between CONV and FLASH





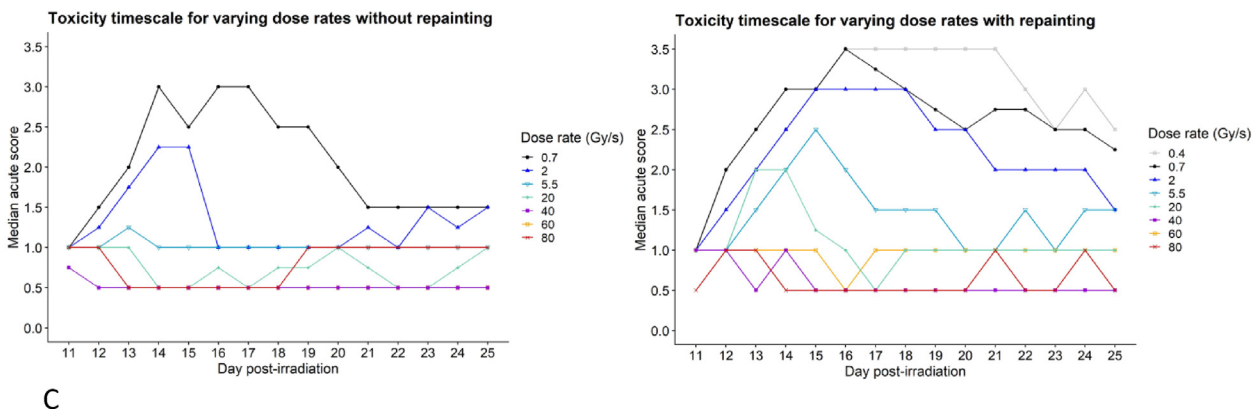


Fig. 3. Dose rate dependency of acute skin toxicity. Comparison of 5 levels of acute damage to the skin at different dose rates using a constant dose of 39.3 Gy. Skin reaction is scored categorically, where scores of 1.5 and 2.0 represent a milder skin reaction; 2.5, a medium skin reaction; and 3.0 and 3.5, severe skin toxicity. (A) The percentage of mice with the reported score at any time during follow-up. Dose was delivered without repainting. (B) The percentage of mice with the reported score at any time during follow-up. Dose was delivered with repainting. The highest possible repainting number is used. Error bars represent variation in the delivered dose rate. Significant difference between CONV (0.37 Gy/sec with repainting) and the given dose rate is indicated with (*), where * indicates $P < .05$, ** indicates $P < .01$, *** indicates $P < .001$ and **** indicates $P < .0001$. (C) Time dependency of acute toxicity. For each treatment group, the points represent the median value of the skin reactions in groups on the specified day.

(Fig. 1), it can be seen that the largest separation between the CONV and FLASH was for dose groups receiving close to 39.3 Gy. Here, severe skin toxicity was observed in all mice receiving CONV irradiation, while FLASH only conferred a small risk of mild toxicity. Therefore, this dose was applied in the current experiments.

A total of 109 mice were included in experiment 1 in 7 dose rate groups in the range of 0.66-79.3 Gy/s, with 7 to 25 mice per dose rate group (Table 1). The mice in experiments 1 and 2 were treated in 3 independent rounds that included a total of 15 treatment groups (Table E1). Round 1 included 7 treatment groups planned with a large spread of dose rates to determine whether additional treatment groups were needed. Rounds 2 and 3 added more mice to the existing treatment groups, as well as more treatment groups. Only 1 treatment group (experiment 2, repainting, 80 Gy/s) only included mice treated in 1 of the 3 rounds. All treatment groups in experiment 1 included mice treated in at least 2 of the 3 independent rounds (Table E1 displays the chronology of the included treatment groups, as well as the number of mice treated in the individual rounds). Skin toxicity data, where a score of 1.5 represents the mildest skin reaction that can be robustly reported, and a score of 3.5 represents severe skin toxicity, are shown in Figure 3A. The mice were scored daily from day 11 to 25 days after treatment, the maximum score at any day during the follow-up was determined, and the percentage of mice in each treatment group with the given score was calculated.

One hundred percent of the animals reached scores of 1.5 and 2.0, indicating mild skin toxicity, at the dose rate of 0.7 Gy/s, with a gradual decrease in the percentage of mice with toxicity between 2 Gy/s and 39.4 Gy/s, and the dose $rate_{50}$ values were 23 Gy/s and 9 Gy/s, respectively (Table 2). For a score of 2.5, representing intermediate skin toxicity, a gradual decrease in toxicity was observed between 0.7 Gy/s and 19.3 Gy/s, with a dose $rate_{50}$ value of 6 Gy/s. For scores of 3.0 and 3.5, indicating severe skin toxicity, there was a decrease in toxicity already between 0.7 Gy/s and 2 Gy/s, with dose $rate_{50}$ values of 1 Gy/s for the score of 3.0 and below 0.7 Gy/s for the score of 3.5.

In the previous studies, the lowest possible dose rate, 0.37 Gy/s, was obtained by delivering a dose of ~39.3 Gy with 320 repaintings of the field. In Experiment 2, to compare how this repainting could potentially influence the FLASH effect, the same field dose rates as above were investigated, still using a constant dose of 39.3 Gy, but with repainting. A total of 86 animals were included in 8 dose rate groups in the range of 0.37 to 88.8 Gy/s, with 7 to 25 animals per dose rate group (Table 1). The pattern of skin toxicity in experiment 2 (Fig. 3B) followed what was observed in mice irradiated without repainting in experiment 1 (Fig. 3A), but with a consistently higher toxicity incidence observed for the same field dose rate with repainting compared with that without repainting. This shifted the dose rate response curves to the right, meaning that a higher dose rate was needed to obtain the same biologic response when the beam was delivered with repainting. The dose $rate_{50}$ value obtained with repainting for scores of 1.5 and 2.0, mild skin toxicity, were 55 and 39 Gy/s, respectively (Table 2), with a gradual decrease in the percentage of mice with toxicity between 5.5 and 89 Gy/s. For a score of 2.5, intermediate skin toxicity, a gradual decrease in toxicity was observed between 2 Gy/s and 40 Gy/s, with a dose $rate_{50}$ value of 14 Gy/s. For scores of 3.0 and 3.5, severe skin toxicity, there was a decrease in toxicity between 0.37 Gy/s and 20 Gy/s, with dose $rate_{50}$ values for a score of 3.0 of 3 Gy/s.

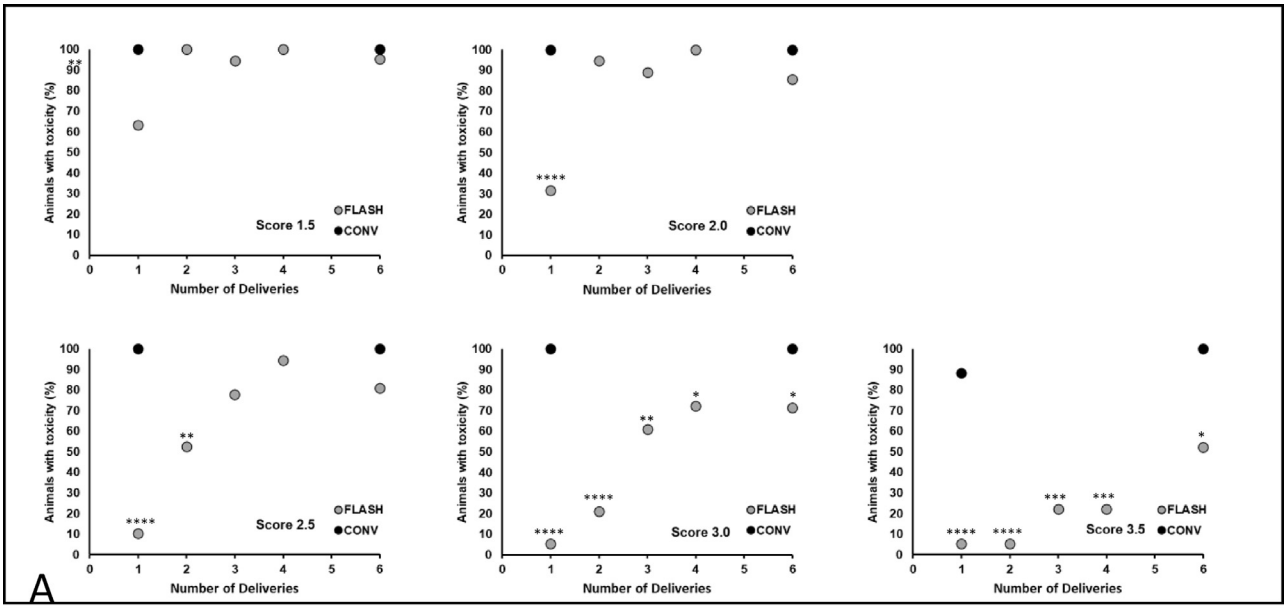
The time dependency of the skin toxicity level is shown in Figure 3C. This shows the onset of the skin reaction (day 11-12), and the initial slope of the curve is independent of the treatment. The time to reach the maximum level, the value of the maximum level, and the time duration before the reaction was reversed depended on the treatment and was seen 13 to 21 days after treatment.

In experiment 3, the influence of splitting the total delivered dose into smaller deliveries with 2 minutes pauses was investigated to simulate delivery with several overlapping fields. The dose was split into equal size deliveries, with either 1 × 39.3 Gy, 2 × 19.7 Gy, 3 × 13.1 Gy, 4 × 9.8 Gy, or 6 × 6.6 Gy deliveries. The same total physical dose as in Experiments 1 and 2, 39.3 Gy, was used, and the field dose rate was fixed at 60 Gy/s. This was based on the dose rate study without repainting (Experiment 1), as we observed the maximal FLASH effect at 60 Gy/s, and the dose rate could technically be controlled to a larger extent than by using the maximum requested beam current of 215 nA. As control, 2 groups of mice were irradiated with CONV (0.37 Gy/s, 320 repaintings, 13-17 mice per group), where 1 group received the dose in 1 delivery, as in previous experiments, and the other group received the dose in 6 × 6.6 Gy with 2-minute pauses. The mice in experiment 3 were treated in 3 independent rounds, with mice treated with CONV included only in rounds 1 and 2, as additional mice in these groups were judged redundant owing to clear results after round 2 (Table E1). As can be seen in Figure 4, splitting the dose did not have an effect on mice irradiated with CONV, and in accordance with previous experiments, 100% of the mice displayed toxicity for most of the toxicity levels. At 60 Gy/s, the single-delivery group revealed the same toxicity as had been seen in previous data (Fig. 3A),

Table 2 Estimated DR_{50} values at the different toxicity levels

Toxicity level	Without repainting DR_{50} (Gy/s)	With repainting DR_{50} (Gy/s)
1.5	23	55
2	9	39
2.5	6	14
3	1	3
3.5	<0.7	2

Animals were irradiated either with or without repainting. Dose $rate_{50}$, the dose rate where 50% of the animals display toxicity at the given level at the used dose.



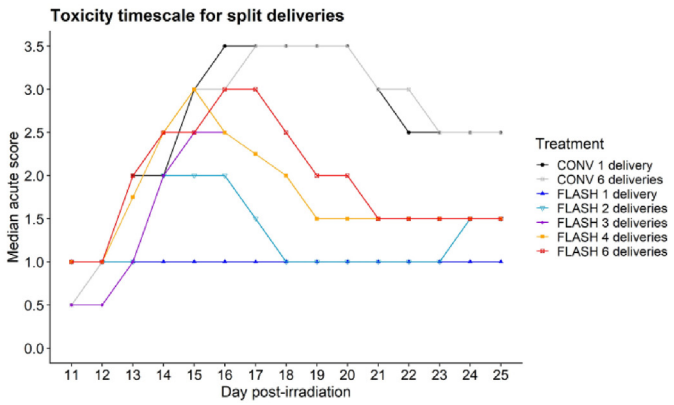


Fig. 4. FLASH effect with split doses. Comparison of 5 levels of acute damage to the skin using a constant dose of 39.3 Gy. Skin reaction is scored categorically, where scores of 1.5 and 2.0 represent a milder skin reaction; 2.5, a medium skin reaction; and 3.0 and 3.5, severe skin toxicity. Dose was delivered at either the CONV dose rate (0.37 Gy/s) or FLASH dose rate (60 Gy/s) applied with 1 or more pauses of 2 minutes. (A) The percentage of mice with the reported score at any time during follow-up. Significant difference between CONV 1 delivery and the given dose rate is indicated with (*), where * indicates $P < .05$, ** indicates $P < .01$, *** indicates $P < .001$ and **** indicates $P < .0001$. (B) Time dependency of acute toxicity. For each treatment group, the points represent the median value of the skin reactions in groups on the specified day.

with only 1 out of 22 animals displaying severe toxicity (score 3-3.5), and around 50% of the animals displaying mild toxicity (score 1.5). Splitting the dose into 2 × 19.7 Gy resulted in an increased toxicity at most levels and splitting the dose into 3 × 13.1 Gy demonstrated an even greater increase in toxicity. For 4 splits (4 × 9.8 Gy), the toxicity reached a plateau, which was at the same level of toxicity as CONV for the lower levels, but at the higher toxicity levels, a FLASH sparing effect was still observed with 4 and 6 deliveries.

The time dependency of the skin toxicity level with split doses is shown in Figure 4B. Here, onset of the skin reaction, if any, occurred around day 11 to 12, independent of treatment. Time to reach the maximum level, level, and duration before the reaction reversed depended on the treatment and were seen 13 to 21 days after treatment.

Discussion

In this study, we used a validated setup and in vivo murine model for evaluating the FLASH effect on normal tissue damage when modulating the time structure of dose delivery. The setup and model are very useful for this type of analysis, owing to a reproducible and very robust outcome, and the study was based on previously obtained dose

response curves for both FLASH and CONV. The aim was to study the dose rate dependency of the FLASH effect using PBS proton beams, as well as the effect of splitting the dose into smaller deliveries, simulating the effect of multiple overlapping fields. It was demonstrated that the dose rate that induced a FLASH effect was dependent on the dose sensitivity of the endpoint. Furthermore, splitting the total dose into more deliveries compromised the FLASH effect.

The mouse foot skin assay represents a well-established model employed in the examination of radiation effects. In this model, early skin reactions are assessed in accordance with a well-defined scoring system.[12,13] Early skin reaction serves as a precisely defined endpoint within classical radiobiology. The underlying biologic mechanisms governing skin erythema have been comprehensively documented.[16] It is characterized by a reddening of the skin at the milder end of the severity spectrum and by the occurrence of moist desquamation at the more severe end of the scale. Temporal dynamics are distinctly defined, with the primary erythema wave manifesting around the 10th day after treatment and peaking approximately on the 14th day. Importantly, the maximum level of the score has a direct correlation with the radiation dose administered. The dominating process behind the observed phenomenon of moist desquamation is due to epidermal cell death, and the subsequent reduction in epidermal cellularity and cell layers leads to a diminished epidermal thickness.

Evaluating the dose rate response in terms of acute toxicity in mouse legs demonstrated no apparent dose rate threshold for inducing a FLASH effect. Rather, the FLASH effect was observed over the whole range of dose rates and was highly dependent on the dose sensitivity of the toxicity level. This meant that at toxicity levels with a lower D50 (dose to induce toxicity in 50% of the animals), a higher dose rate was required to observe tissue sparing, whereas at toxicity levels with higher D50, a reduced toxicity was already observed at the lowest dose rates, from 2 to 5.6 Gy/s. Hence, depending on the dose and on the endpoint used, even a slight increase in the dose rate could lead to a significant reduction of severe toxicity. For clinical implementation and FLASH treatment plan optimization, this may imply that escalating low dose rates to moderate dose rates is more advantageous than increasing high dose rates to a greater degree.

Changing the time structure of the delivered dose by introducing repainting of the field while keeping the field dose rate constant led to an overall shift in the dose rate response curves to the right, meaning that a higher dose rate was needed to induce a FLASH effect (Fig. 3A and B).

The current data indicate that for the used dose, depending on the endpoint, the maximum FLASH effect was reached at 60 to 80 Gy/s (field dose rate), which is the limit for our clinical proton facility. This was supported in a modeling approach of the current data, using a common formalism based on an oxygen enhancement ratio weighted biologic dose.[17] However, to validate if this is a consistent phenomenon, comparable experiments with higher doses and even higher dose rates——for example, using electron beam FLASH——could demonstrate if a greater FLASH effect could be obtained. Depleted oxygen as the mechanism behind the FLASH effect is however highly debated, since certain analyses indicate that the oxygen depletion is too small to reach radioprotective levels.[18–22]

The data in this study demonstrate that a FLASH effect for the current endpoint can be seen at lower field dose rates down to below 1 Gy/s, which is lower than what has been reported so far, where a threshold of 40 Gy/s has been often mentioned.[7,23–26] This is pointing toward the importance of the reference dose rate of CONV in FLASH studies. In 1 of our previous studies, with the aim of quantifying the FLASH effect, the used CONV dose rate was 0.37 Gy/s, and here, a FLASH factor of 1.44-1.58 was found.[9] If a higher CONV dose rate had been used, the observed outcome FLASH factor would have been lower. In the present study, a FLASH effect was observed both with and without repainting at dose rates of 5.6 Gy/s, where the overall treatment time was 7 seconds. This is promising for potential clinical use of FLASH with treatments extending for several seconds.

The data in the present study demonstrate that a dose rate effect depends both on the dose rate and the dose sensitivity of the endpoint, indicating that different conclusions can be reached for different combinations of these factors. This suggests that care should be taken when concluding anything on dose rate effects based on single-dose and single-endpoint studies. This can explain the observed difference in 2 previous studies addressing the influence of dose rate on the FLASH effect. In these studies, the endpoints of either recognition ratio 2 months post 10 Gy whole brain irradiation[2] or crypt survival 3.75 days post 11.2 or 12.5 Gy whole abdominal irradiation[6] were used. In the first study, an induction of the FLASH sparing effect was observed at an average dose rate of 30 Gy/s, and the maximal FLASH effect was observed at 100 Gy/s, whereas in the latter study, average dose rates of at least 280 Gy/s were necessary for a significant FLASH sparing effect on crypt survival, and the highest sparing was found at the highest dose rate, using a single pulse delivery.

To further study the effect of modulating the time structure of the delivered dose and to simulate the effect of delivering multiple overlapping fields, the same overall dose was delivered in 1 to 6 deliveries, keeping the dose rate constant. It was observed that splitting the total dose into 2 deliveries compromised the FLASH effect. Additionally, depending on toxicity level and hence D_{50} of the endpoint, splitting the dose into more than 3 deliveries abolished the FLASH effect. For the higher toxicity levels, with a higher D_{50}, the number of animals displaying toxicity reached a plateau at 4 to 6 deliveries. There still appeared to be a FLASH effect, as the skin toxicity was lower than that for the animals treated with CONV. The CONV arm was 0.37 Gy/s. Using a CONV reference with a higher dose rate could have led to the conclusion of an abolishment of the FLASH effect for 6 deliveries. The finding that splitting the total dose compromises the FLASH effect is consistent with data from a recent study by Mascia et al,[27] where it was demonstrated that the FLASH sparing effect at 30 and 35 Gy was

abrogated when 2 pauses of 2 minutes were included in the dose delivery.

Decreasing the dose rate and introducing beam pauses compromised the FLASH effect to different degrees across the included toxicity level endpoints. In both experiment types, the highest toxicity level, score 3.5, displayed a FLASH effect both at very low dose rate and with 5 introduced beam pauses. If assuming the FLASH effect to be a dose-modifying factor, both modulations are decreasing this factor, which is the difference between CONV and FLASH, leading to an increased dose sensitivity for the FLASH treatment. As the score 3.5 endpoint has the highest D_{50}, it seems intuitive that this endpoint is the last to be affected by the increasing dose sensitivity. The observation that a higher dose rate is required for more radiosensitive structures can also offer an explanation for the difference in the FLASH effect previously observed in different tissues, where skin toxicity has demonstrated a much larger FLASH effect than gut toxicity,[24] for example. However, when comparing acute to late toxicity, the higher radio resistance does not seem to lead to an increased FLASH effect.[10,28] The toxicity variations with changing dose rates, repainting, and beam splits within 1 single endpoint may be explained by a common formalism based on an oxygen enhancement ratio weighted biologic dose.[17]

For all 3 studies, the time dependency of the acute toxicity followed the classical radiobiology, as described earlier in this section, with the time to the onset of the reaction being independent of the treatment. Conversely, the time to reach the maximum, the level, and the duration before the reaction reversed were dependent on the treatment. The pattern of the time dependency in the different treatment groups followed the previously observed dose dependency,[11,13] confirming that FLASH is acting as a dose-modulating factor.

The model and experimental design used here is very specific to acute radiation injury induced by exposure to high doses of irradiation, and as such, is a mechanistic model of radiation injury. For a clinical translation of FLASH, other factors, such as fractionation, need to be considered and experimentally tested. Nevertheless, the model provides rich information regarding the degree of tissue sparing for a wide range of beam time patterns, which may be helpful in elucidating the FLASH effect. The current data are restricted to acute effects, and it is not possible to predict from here whether the late effects on normal tissue will follow the same patterns.

In conclusion, the FLASH effect on normal tissue toxicity is sensitive to a range of parameters, including dose rate, time structure of the beam, and beam pauses. The FLASH effect observed in experimental studies is furthermore very sensitive to the dose sensitivity of the chosen endpoint and the dose rate of the reference radiation.

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