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[Article Full Title]
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[Short Running Title]
Hypofractionated FLASH vs CONV in a glioma model

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ABSTRACT

Introduction: To ensure a clinical translation of FLASH radiation therapy (FLASH-RT) for a specific tumor type, studies on tumor control and toxicity within the same biological system are needed. In this study, our objective was to evaluate tumor control and toxicity for hypofractionated FLASH-RT and conventional radiation therapy (CONV-RT) in an immunocompetent rat glioma model.

Materials and Methods: Fisher 344 rats (N=68) were inoculated subcutaneously with NS1 glioma cells and randomized into groups (n=9-10 per group). CONV-RT (~8 Gy/min) or FLASH-RT (70-90 Gy/s) was administered in three fractions of either 8 Gy, 12.5 Gy, or 15 Gy using a 10 MeV electron beam. The maximum tumor diameter was measured weekly and overall survival was determined until day 100. Long-term tumor control was defined as no evident tumor on day 100. Animals were evaluated for acute dermal side effects at 2-5 weeks after completed radiation therapy, and for late dermal side effects at 3 months after initiation of treatment.

Results: Survival was significantly increased in all irradiated groups compared to control animals ($p<0.001$). In general, irradiated tumors started to shrink at one week post completed radiation therapy. In 40% (23/58) of the irradiated animals, long-term tumor control was achieved. Radiation-induced skin toxicities were mild and consisting of hair loss, erythema and dry desquamation. No severe toxicity was observed. There was no significant difference between FLASH-RT and CONV-RT in overall survival, acute side effects, or late side effects for any of the dose levels.

Conclusions: This study shows that hypofractionated FLASH-RT results in long-term tumor control rates similar to CONV-RT for the treatment of large subcutaneous glioblastomas in immunocompetent rats. Neither treatment technique induced severe skin toxicity.

Consequently, no significant difference in toxicity could be resolved, suggesting that higher doses might be required to detect a FLASH sparing of skin.

INTRODUCTION

Glioblastoma is a highly aggressive primary brain tumor associated with a short median survival. The development of effective treatment protocols against glioblastoma is a challenge. The standard protocol introduced by Stupp et al. includes maximal safe tumor resection followed by radiation therapy administered as 60 Gy in 30 fractions, 5 days per week, with concomitant and adjuvant temozolomide. With this protocol, median survival in study patients increased from 12.1 months with radiation therapy alone to 14.6 months with the addition of temozolomide [1]. Despite aggressive treatment, glioblastoma tumors are highly resistant [2]. Compared to patients who qualify for study inclusion, patients in population based series have been shown to have worse overall survival, in many cases less than one year [3].

Radiation therapy is one of the few treatments that has provided glioblastoma patients with a survival benefit. However, a limiting factor is the radiation-induced side effects, including neurocognitive decline. With efforts to increase the absorbed dose to a therapeutic level, severe toxicity arises in sensitive areas of the brain. Therefore, any approach that could improve the therapeutic index by increasing normal tissue tolerance would improve the benefits of radiation therapy, allowing increased dose to the tumor to improve tumor control. Contemporary radiation therapy techniques, such as hypofractionated radiation therapy and stereotactic radiosurgery, are strategies that could offer shorter treatment courses to maximize quality of life and allow for dose intensification for improved tumor control [4]. Still, major problems regarding radiation therapy against glioblastoma are both inherent resistance and further development of adaptive radioresistance [5].

In 2014, a novel approach to broaden the therapeutic window was proposed. By reducing the beam-on time from several minutes to a fraction of a second, Favaudon et al. observed significantly less radiation-induced fibrosis in mice lung [6]. The technique of using ultra-high dose rate irradiation was coined FLASH radiation therapy (FLASH-RT). In recent years, a lower toxicity on normal tissue has been confirmed in various animal models and organs [7] as well as in the skin of higher mammals, as seen in a minipig and in cat- and canine cancer patients [8,9]. The protective effect appears to be triggered at average dose rates greater than 30 Gy/s [10]. Furthermore, FLASH-RT has been shown to be equally effective as conventional radiation therapy (CONV-RT) delivered at average dose rates of a few Gy/min, in preventing tumor growth [6,11-16]. Considering the evidence mentioned above, there may be a therapeutic gain with FLASH-RT that can increase the probability of uncomplicated tumor control as compared to CONV-RT.

In brain, the use of ultra-high dose rates has been demonstrated to result in less inflammation as compared to conventional dose rates [17], as well as a higher degree of protection of blood vessels [18] and neuro-cognitive functions in mice [10,11,19]. A few studies have shown that tumor growth delay is similar for FLASH-RT and CONV-RT in xenograft glioma models [11,12]. However, data on how FLASH-RT compares with CONV-RT with respect to long-term tumor control, which is the goal of curative RT, have not yet been published.

In this study, we used a synergetic subcutaneous glioblastoma model with an infiltrative growth pattern [20] in fully immunocompetent animals. The aim was to evaluate and compare tumor control and treatment toxicity for various doses of hypofractionated FLASH-RT vs. CONV-RT by assessing overall survival, tumor growth and long-term tumor control, as well as the frequency of acute and late local dermal toxicity.

METHODS AND MATERIALS

Ethics statement

This study was approved by the animal ethics committee in Lund with permit ID 5-8-18-02383/2020 and amendment 2021. All efforts were made to minimize animal suffering.

Rat glioma NS1 cells

The NS1 rat glioma cell line is a new GFP-positive tumor cell line created in our laboratory [20]. The cell line was initiated by treating pregnant homozygous GFP-positive Fischer 344 rats with ENU, where the offspring subsequently developed GFP-positive CNS-tumors. The NS1 tumor cell line was established from an intraparenchymal tumor growing in the offspring. Rats inoculated with NS1 cells develop cell-rich tumors with an invasive growth pattern. Tumors are positive for GFAP, GFP, and express wt-IDH1 [21]. Sandwich Elisa was used to rule out Mycoplasma infection in cells and supernatant and was used according to the manufacturer's instructions (MycoProbe R&D Systems®). To verify the GFP signal, cells were cultured for 1 to 2 days in two-chamber culture slides (Thermo Fisher Scientific) at 37°C in a humidified incubator with 5% CO2. The medium was removed and the cells were fixed in 4% paraformaldehyde. Cells were mounted with Eukitt Quick-hardening mounting medium (Sigma-Aldrich), stained with Hoechst (Thermo Scientific) and photographed with a fluorescent microscope fitted with the appropriate wavelength filters (Figure 1a). Preparing cells for inoculations, they were cultured using Iscove's Modified Dulbecco's Medium (IMDM) with addition of 1% ml Na-pyruvate, 1% ml HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1% ml gentamycin, as well as 10% inactivated fetal calf serum (heated to 56 °C for 30 minutes). After culturing in T25 flasks, the cells were prepared for inoculation by removal of the medium, and washed gently with PBS. Trypsin (Invitrogen) was added, and cells were incubated in 37°C for 1-2 minutes to detach the adherent cells from the flask. Thereafter, medium was added, and viable cells were counted. The cells were

centrifuged, the supernatant was removed, and the cell pellet was resuspended to achieve the concentration used for inoculation, 1000 cells/µl.

In vivo experiments

Seven to ten weeks old female Fischer 344 rats (Fisher Scientific, Germany) were purchased and housed in pairs in cages (Taconic® type 3 cages, two animals/cage) with access to water and fed *ad libitum* with rat chow. Rats were allowed to acclimatize for one week, after which tumor cells (50 000 NS1 cells) were subcutaneously inoculated in the hindleg at experimental day 0 with the animals in prone position. The estimated mean weight of the animals at the time of inoculation was 120 to 150 g, depending on age. The inoculation was performed during general anesthesia with isoflurane inhalation. At day 18, all tumors had reached a size ≥ 10 mm, but no tumor was > 30 mm, and animals were randomized into different treatment groups. Kruskall-Wallis analysis did not show any difference between the groups (p=1.0) with respect to tumor size at randomization (Figure 1b). N=68 animals were included in the study, with 9 or 10 animals per treatment group (n=10 controls, n=9 FLASH-RT 8 Gy x 3, n=10 FLASH-RT 12.5 Gy x 3, n=10 FLASH-RT 15 Gy x 3, n=10 CONV-RT 8 Gy x 3, n=9 CONV-RT 12.5 Gy x 3, n=10 CONV-RT 15 Gy x 3). Animals in the control group were inoculated with tumor cells without any further treatment. Animals were monitored daily, and those showing signs of paresis, tumor diameter >30 mm, or declined general condition were euthanized. The criteria for euthanasia are defined in the animal ethics permission and in accordance with the acceptance from the ethics board.

Rat irradiation

Radiation therapy was administered in three fractions (day 21, 25 and 28) of either 8 Gy, 12.5 Gy, or 15 Gy, using CONV-RT or FLASH-RT (Figure 1c). The irradiation source was a 10 MeV electron beam from a clinical Elekta Precise linear accelerator (Elekta AB,

Stockholm, Sweden). For FLASH-RT delivery the treatment machine was temporarily modified to deliver ultra-high dose rate electrons in 3.5 µs pulses, as described elsewhere [22]. CONV-RT treatments were delivered during 60-110 seconds with an average dose rate of 8 Gy/min and an instantaneous dose rate of 200 Gy/s (Figure 1d). The FLASH-RT treatments were delivered with 4, 6 or 8 pulses, resulting in total treatment times of ≤180 ms, average dose rates of ≥70 Gy/s, and instantaneous dose rates of $\geq 5.6 \cdot 10^5$ Gy/s. The dose per pulse was adjusted by varying the source-to-surface distance (SSD) between 65 and 67 cm. For both CONV-RT and FLASH-RT irradiation, an electron applicator was fitted with a Cerrobend plate creating a circular radiation field of 3 cm in diameter. Before treatment, the animals were anesthetized by intraperitoneal injection of Ketalar/Rompun and positioned in custom-made PMMA boxes. For irradiation, the boxes were positioned at the corresponding SSD with the tumor in the center of the field (Figure 1e). The absorbed dose was prescribed at 4 mm depth in the animal. Dosimetry was performed using GafChromic film (XD film, Ashland Advanced Materials, Bridgewater NJ) in a polystyrene phantom placed in one of the boxes to mimic the rat, measuring percentage depth dose curves and dose profiles at 4 mm depth for both the CONV-RT and FLASH-RT beam (Figure 1f). XD film measurements at 4 mm depth in the phantom were also performed prior to each treatment session. During treatment, a Farmer-type ionization chamber (NE 2505/3-3A) positioned in a custom-made holder was used for relative output measurements to ensure output stability in FLASH-RT mode.

Tumor growth, overall survival and treatment response

The maximum tumor diameter (*d*) was measured for all animals once every week using a caliper. Measurements were carried out by the same personnel throughout the study, blinded to the assigned treatment group. Overall survival was determined from the day of inoculation (day 0) until the criteria for euthanasia was reached. At the end of the study, animals were further divided by treatment response based on the maximum tumor diameter at day 100

(d_{100}), as long-term tumor control ($d_{100} = 0$), stable disease ($0 > d_{100} \leq d_{18}$), tumor progression ($d_{100} > d_{18}$) or animals euthanized during the observation period due to large tumor.

Acute and late radiation induced skin reactions

Animals were evaluated for acute radiation-induced skin reactions according to a phenotypic grading scale 1-6 (1: normal, 2: hair loss, 3: erythema, 4: dry desquamation, 5: <30% moist desquamation, and 6: >30% moist desquamation) established by de Andrade and colleagues [23]. Observations and toxicity evaluations were performed weekly, 2-5 weeks after completed radiation therapy. Animals that were euthanized due to large tumors (diameter >30 mm) during the study period were excluded from the analysis to avoid mistaking a subtherapeutically treated fast-growing tumor for local side effects to the skin. At three months after initiation of radiation therapy, the surviving animals were evaluated for late skin toxicity by determining the ratio of animals with toxicity >grade 1.

Statistical analysis

SPSS was used for statistical evaluations. Kruskall-Wallis test, Mann-Whitney U-test, and Fisher's Exact test were performed for non-parametric analyses. Survival curves were assessed using Log Rank test. All significance tests were performed with a significance level of 5%.

RESULTS

Dose delivery

The measured beam characteristics demonstrated a FWHM of 3.1 cm for both the FLASH-RT and CONV-RT beams and a therapeutic range of 2.4 cm (Figure 1f), indicating that the animals were irradiated with good dose coverage across the tumor. Based on the film measurements conducted prior to each treatment session, all fractions were estimated to be within 5% of the prescribed dose and 81% (141/174) of the fractions were within 3%. The average agreement between the prescribed dose and the estimated dose to the tumor was 0.1% (range -0.9% to +1.3%) for CONV-RT delivery and 2.5% (range -0.4% to +5.0%) for FLASH-RT delivery.

Tumor growth and overall survival

In general, irradiated tumors started to shrink at one week post completed radiation therapy (Figure 2). Mean tumor size for each group (solid lines in Figure 2) was determined at each measuring point until the first animal in the respective group was euthanized. All animals in the control group were euthanized prior to day 100 due to tumors exceeding 30 mm in diameter.

Survival was significantly increased in all groups compared to control animals (Log Rank test, $p<0.001$) (Figure 3a-d). There was no statistically significant difference between animals treated with FLASH-RT and CONV-RT at any of the dose levels. For CONV-RT, there was a statistically significant difference comparing 8 Gy x 3 versus 12.5 Gy x 3 ($p=0.007$) or 8 Gy x 3 versus 15 Gy x 3 ($p=0.026$), but no difference comparing 12.5 Gy x 3 versus 15 Gy x 3. For FLASH-RT, there was a statistically significant difference comparing 8 Gy x 3 versus 15 Gy x 3 ($p=0.012$), but no difference comparing 8 Gy x 3 versus 12.5 Gy x 3, or 12.5 Gy x 3 versus 15 Gy x 3. With the present sample size, power estimation was 0.999 to detect differences in survival between groups (significance level 5%).

Treatment response

In total, 78% (45/58) of the irradiated animals were alive on day 100 and in 40% (23/58) of the tumors a long-term tumor control was achieved by radiation therapy. For animals irradiated with 8 Gy x 3, a similar tumor control was observed for CONV-RT and FLASH-RT, with 44% (4/9) and 40% (4/10) of animals either with long-term tumor control or stable disease on day 100, respectively (Figure 3e). For animals irradiated with 12.5 Gy x 3, two animals in the FLASH-RT group were euthanized before the end of the study due to large tumors, while long-term tumor control or stable disease was evident for all animals in the CONV-RT group. All animals irradiated with 15 Gy x 3 achieved long-term tumor control or had stable disease, except for one animal belonging to the CONV-RT group, which was euthanized one week prior to the end of the observation period due to a large tumor.

Radiation induced skin reactions

Radiation-induced skin reactions were generally mild and consisting of hair loss, erythema and dry desquamation. Acute skin effects at 2-5 weeks post completed radiation therapy were dose- and time-dependent (Figure 4a). There was no significant difference in acute side effects between FLASH-RT and CONV-RT for any of the investigated dose levels at any of the investigated time points (Mann Whitney U-test). Most acute side effects healed spontaneously. The ratio of survivors with late side effects >grade 1 at three months post initiation of radiation therapy increased with increasing fraction dose (Figure 4b). There was no significant difference in late side effects between CONV-RT and FLASH-RT for any of the dose levels investigated (2-sided Fisher's Exact test).

DISCUSSION

In the present study, we compare hypofractionated FLASH-RT vs. hypofractionated CONV-RT for treatment of large subcutaneous glioblastomas in immunocompetent rats. All animals had verified tumors upon initiation of treatments. Treatment doses were chosen to achieve

tumor growth delay as well as high probability of long-term tumor control for the highest doses. To obtain toxicity data on the same material, we examined the animals for normal tissue complications at early time points (weekly, 2-5 weeks post RT) and at a late time point (three months post RT).

Despite recent *in vitro* findings of a sparing of tumor cells using FLASH-RT [24], the current study showed no difference in survival between CONV-RT and FLASH-RT for any of the delivered doses. Similar as previous studies on glioblastoma-bearing mice [11,12], irradiated animals displayed a delayed tumor growth as compared to control animals. At doses of 8 Gy x 3, neither FLASH-RT nor CONV-RT was sufficient to achieve adequate tumor control in all animals. However, at higher dose levels most of the animals achieved long-term tumor control or had stable disease on day 100. On the contrary, Montay-Gruel et al. showed that no animals with glioblastoma in the brain, treated with 10 Gy x 3 at three days post inoculation in immunodeficient animals, lived for 100 days [11]. Local early side effects were time-dependent, indicating that the time elapsed from irradiation to evaluation appears important and that frequent follow-up is needed. There was no severe toxicity associated with any of the treatments. No difference in acute or late side effects between FLASH-RT and CONV-RT could be resolved for any of the investigated dose levels. Combining these results with previous studies comparing skin toxicity between FLASH-RT and CONV-RT [8,13,25-27], it seems that high fraction doses are required to detect a FLASH sparing effect in the skin. In the first report on a skin sparing effect of FLASH-RT compared to CONV-RT, single fractions in the range 28-34 Gy were administered to the skin of a pig [8]. Soto et al. found a lower incidence and severity of skin ulcerations for FLASH-RT compared to CONV-RT at doses of 30 and 40 Gy, but no severe toxicity ≤20 Gy [25]. In a direct comparison of 15 Gy FLASH-RT and 15 Gy CONV-RT in a human patient with multiply relapsed cutaneous T-cell lymphoma, Gaide et al. did not observe any difference in acute or late effects [26]. Furthermore, our preliminary results on skin toxicity in flank melanoma-bearing mice show a substantial difference between FLASH-RT and CONV-RT in a single fraction of 25 Gy, as

compared to no or a small difference for doses in the range 10-20 Gy (unpublished). It should be noted that a large subtherapeutically treated tumor on the flank may influence the scoring of local dermal side effects. To avoid this in the present study, animals that were euthanized due to large tumors during the study period was excluded from the toxicity analysis. At the timepoint for evaluation of late side effect, the animals had either no tumors or small tumors, implying that the evaluation was not compromised by tumor size. However, separate studies of toxicity in tumor-free animals are needed to completely avoid the tumor as a confounding factor.

In contrast to skin tissue, Montay-Gruel et al. demonstrated improved neuro-cognitive function in nude mice following brain irradiation with hypofractioned 10 Gy x 3 FLASH-RT as compared to CONV-RT [11]. Therefore, it is likely that the threshold dose for inducing a FLASH sparing effect varies between normal tissue types and the environment. For example, it has been shown *in vitro* that the FLASH effect depends on oxygen concentration [28]. Also, the use of immunocompromised animals may result in a different response than immunocompetent hosts. It is known that radiation therapy has immunological effects that can reshape the tumor microenvironment [29] and it has been proposed that the FLASH effect can be caused by a modification of the immune response, as the ratio of irradiated circulating T-lymphocytes is likely to be reduced compared to the longer treatment time used for CONV-RT [30]. Because of this, we believe that it is important to investigate the FLASH effect in immunocompetent animal models.

The measured beam characteristics indicate that the animals were irradiated with adequate dose coverage across the tumor, and the absorbed dose measurements performed prior to each treatment session confirmed that the prescribed doses were delivered accurately. For FLASH-RT, the temporal structure of the electron beam has been shown to be important [7,11,12]. In this study, the treatment parameters previously recognized as critical for the FLASH effect, i.e., average dose rate, instantaneous dose rate, beam-on time and fraction dose, were expected to be sufficient to observe a potential FLASH effect [7,11,12]. Using

similar temporal parameters, we have observed a sparing FLASH effect in our laboratory, both *in vitro* [24,28] and *in vivo* (manuscript in process). It could be that the fraction doses investigated in this study are too low to observe a sparing effect on skin. However, long-term tumor control was still achieved. Further studies on tumor cure and normal tissue toxicity are required to investigate the therapeutic window at higher doses. FLASH-RT has not yet been explored for standard fractionated treatments, such as the 60 Gy/30 fractions used in the Stupp protocol currently employed for glioblastoma patients. However, as discussed above, there are indications that higher fraction doses are required to observe a FLASH effect. Ultimately, to ensure a clinical translation of FLASH-RT for a specific tumor type and site, the therapeutic window should be studied in clinically interesting scenarios in models where tumor cure can be achieved.

Although the subcutaneous glioma model cannot be used to draw conclusions about the interactions between the microenvironment in the brain and glioma cells, it is used here as a first step to investigate tumor control and normal tissue complications in multiple treatment groups. In the intracranial setting, additional challenges are encountered, including the blood-brain barrier which facilitates immune evasion, as well as spread of tumor cells within the brain parenchyma and interactions with the complex microenvironment [31]. In the next step, we will use the glioma model intracranially to further explore the impact of these issues in immunocompetent animals treated with FLASH-RT and CONV-RT.

CONCLUSION

In the present study, we show that long-term tumor control can be achieved in large subcutaneous glioblastomas without inducing severe skin toxicity, using both hypofractionated FLASH-RT and CONV-RT. No difference in tumor response between FLASH-RT and CONV-RT could be resolved, nor any significant difference in treatment

toxicity, suggesting that higher doses might be required to detect a FLASH sparing of the skin. This study is the first to show that there is no difference in long-term tumor control rates between FLASH-RT and CONV-RT in immunocompetent glioblastoma-bearing animals.

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FIGURE CAPTIONS

Figure 1: Study design to compare hypofractionated FLASH-RT and CONV-RT in a subcutaneous rat glioma model. (a) The rat glioma cell line NS1 is a GFP-positive tumor cell line (left panel). Tumor cells are marked with Hoescht nuclear staining (right panel) and the GFP autofluorescence can be detected (middle panel). (b) Animals were randomly assigned between groups (n=9-10) according to tumor size on day 18 after inoculation, and a Kruskall-Wallis analysis did not show any statistical difference between the groups. The Box and Whiskers plot presents the resulting distribution of tumor size across groups. (c) Timeline of the study design. Animals were inoculated in prone position on day 0 and irradiated with either CONV-RT or FLASH-RT on day 21, 24 and 28. (d) Treatment parameters for CONV-RT and FLASH-RT. (e) For irradiation, an electron applicator with source-to-applicator-end distance 65 cm was attached to the gantry head of a clinical linear accelerator and fitted with a Cerrobend plate for beam collimation. Rats were placed in PMMA boxes and positioned one-by-one in close connection to the Cerrobend plate. (f) Dose profiles at 4 mm depth and percentage depth dose curves for CONV-RT (red) and FLASH-RT (blue) measured in a polystyrene phantom placed inside a PMMA box to mimic the rat irradiation setup. This figure was created with BioRender.com.

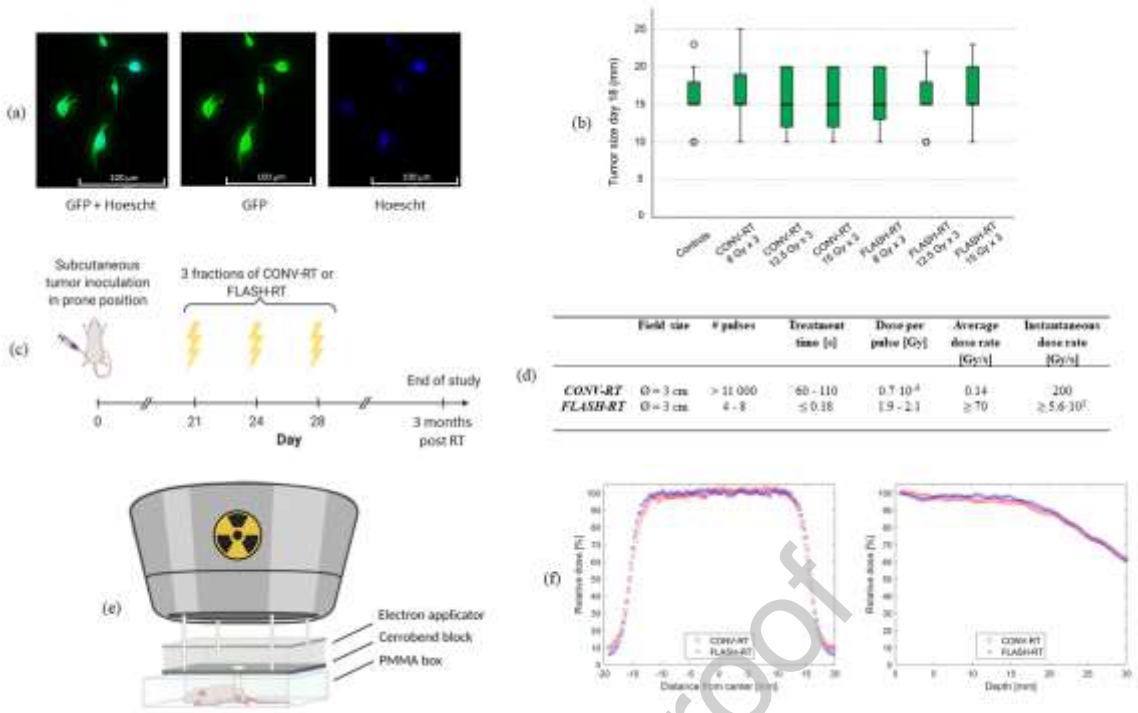


Figure 2: Tumor growth for subcutaneous glioblastoma inoculated at the flank of Fisher 344 rats and irradiated with hypofractionated CONV-RT or FLASH-RT at day 18, 21 and 24, as well as for non-irradiated controls. Dots represent tumor size for each individual animal measured once a week. Calculated mean tumor size for each group is presented as solid lines. Dotted lines indicate the mean tumor size calculated when one or more animals had been euthanized during the study period, thus slightly underestimating the 'actual' mean tumor size.

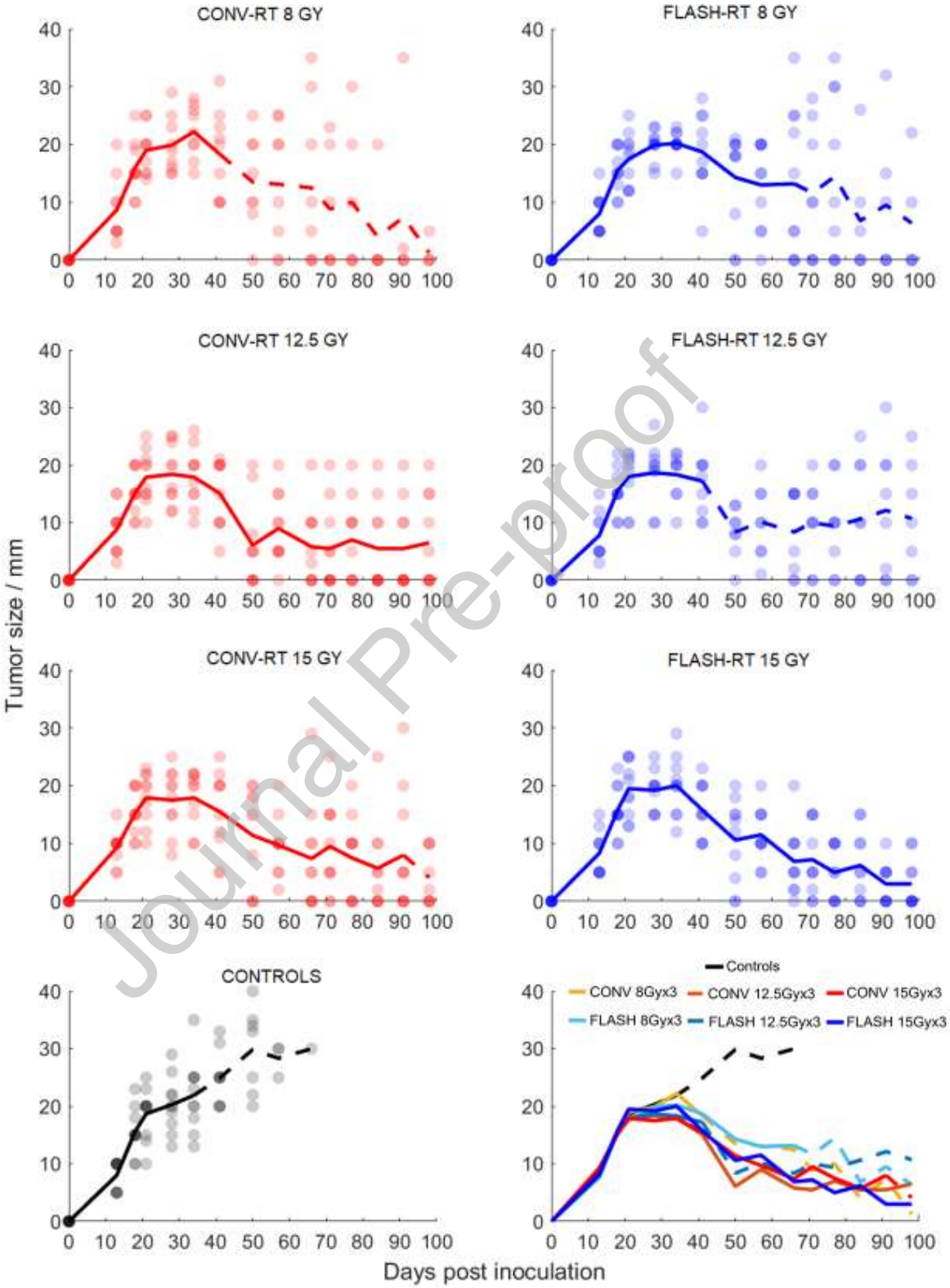
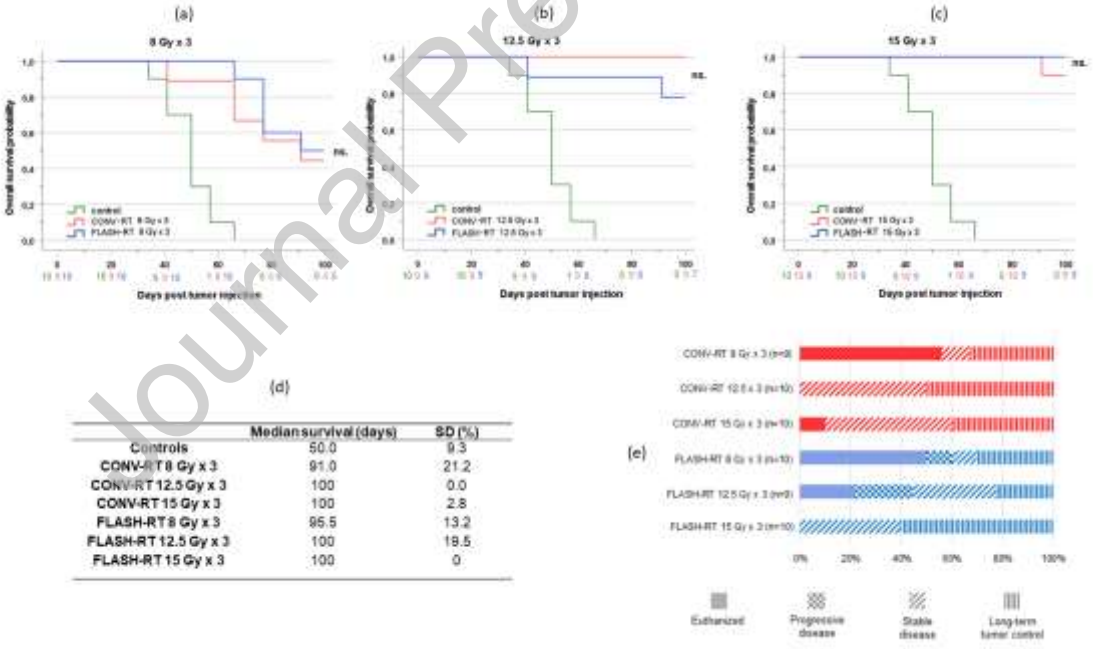


Figure 3: Overall survival and treatment response for glioblastoma-bearing rats irradiated with hypofractionated CONV-RT or FLASH-RT, as well as non-irradiated controls. (a-c) Kaplan-Meier survival curves for all study groups, demonstrating no significant difference in survival between CONV-RT and FLASH-RT at any of the investigated dose levels (n=9-10, Log Rank test). No deaths occurred after CONV-RT 12.5 Gy x 3 and FLASH-RT 15 Gy x 3. Colored numbers below the x-axis represents the number of animals still alive at each time point. (d) Median survival and standard deviations for each study group. (e) Animals irradiated with hypofractionated CONV-RT or FLASH-RT categorized on day 100 as euthanized (dotted pattern), progressive disease (check), stable disease (diagonal stripes) or long-term tumor control (vertical stripes). In total, 78% (45/58) of the irradiated animals were alive on day 100.



	Median survival (days)	SD (%)
Controls	50.0	9.3
CONV-RT 8 Gy x 3	91.0	21.2
CONV-RT 12.5 Gy x 3	100	0.0
CONV-RT 15 Gy x 3	100	2.8
FLASH-RT 8 Gy x 3	95.5	13.2
FLASH-RT 12.5 Gy x 3	100	18.5
FLASH-RT 15 Gy x 3	100	0

Figure 4: Severity of skin toxicity for animals irradiated with CONV-RT or FLASH-RT in three fractions of either 8 Gy, 12.5 Gy or 15 Gy. Local dermal side effects were graded on a scale 1-6 (1: normal, 2: hair loss, 3: erythema, 4: dry desquamation, 5: <30% moist desquamation, and 6: >30% moist desquamation). No severe toxicity (grade >4) was observed. There was

no significant difference in acute or late skin toxicity between CONV-RT and FLASH-RT for any of the dose levels at any time point (Mann-Whitney U-test and 2-sided Fisher's Exact test). (a) Acute skin toxicity at 2-5 weeks post completed radiation therapy. Bars represent the average score for each treatment group and dots represent scores of individual animals. (b) The ratio of survivors with late side effects >1 at 3 months post initiated radiation therapy.