



Short Communication

Comparing radiolytic production of H₂O₂ and development of Zebrafish embryos after ultra high dose rate exposure with electron and transmission proton beams



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ARTICLE INFO

Article history:

Received 25 April 2022

Received in revised form 30 June 2022

Accepted 13 July 2022

Available online 19 July 2022

Keywords:

Electron FLASH

Proton FLASH

Hydrogen peroxide

Zebrafish embryos

ABSTRACT

The physico-chemical and biological response to conventional and UHDR electron and proton beams was investigated, along with conventional photons. The temporal structure and nature of the beam affected both, with electron beam at ≥ 1400 Gy/s and proton beam at 0.1 and 1260 Gy/s found to be isoefficient at sparing zebrafish embryos.

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Radiation therapy is a pillar of anti-cancer treatments. However and despite technological improvement of radiation delivery, the dose required to cure tumors remains close to normal tissue tolerance. To enhance the therapeutic window and overcome radiation-induced normal tissue toxicity, we proposed to irradiate at ultra high dose rate, FLASH-RT. At the preclinical level, FLASH-RT is known to increase normal tissue tolerance while maintaining tumor killing [1]; a biological outcome that was named the FLASH effect, now investigated worldwide with various type of beams.

The FLASH effect has been primarily studied in vivo models with electron beams of intermediate energy [2] and reproduced with other beams including proton beams at 60–160 Gy/s [3–7]. Electron beams are pulsed whereas proton beams are quasi-continuous; this inherent difference in temporal beam structure dictates major differences in instantaneous dose rates that might impact early physico-chemical and biological outcomes. This prompted us to perform a systematic comparison between electron and proton beams at conventional and UHDR. While opera-

tional constraints and uncertainty on the dose prevented us from using the exact same beam parameters impacting dose rates across the experiments, our aim was to be as close as possible to assess dose rate-specific vs beam-specific physico-chemical (radiolytic) and biological (developmental) responses. The radiolytic yields of hydrogen peroxide produced in pure water equilibrated at 4% O₂ to mimic physioxia as well as the early development of zebrafish embryos was investigated upon irradiation with conventional (≤ 1 Gy/s) and UHDR (≥ 1260 Gy/s) electron and proton beams. In ZF, functional and molecular outcomes were investigated including survival, growth as well as cell death and proliferation. A conventional dose rate 225 kV photon beam was also included as a reference.

Material and methods

Full material and method can be found in the [supplementary material](#).

Irradiation devices

Irradiations and dosimetry were performed as already described using [8–13]:

Abbreviations: UHDR, ultra-high dose rate; ZF, zebrafish; DMF, dose modifying factor.

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- 1) Xrad 225CX/225 keV (Pxi Precision X-ray), at 0.037 Gy/s.
- 2) eRT6/Oriatron 5.5 MeV electron beam (PMB-Alcen), at 0.1 Gy/s for conventional dose rate and 100 Gy/s, \geq 1400 Gy/s for UHDR.
- 3) PSI Comet Cyclotron 235 MeV (Gantry 1) in transmission mode, at 0.1, and 0.9 Gy/s for conventional dose-rate, 90 Gy/s and 1260 Gy/s for UHDR

Full beams parameters can be found in [Table sup 1](#).

Water radiolysis experiments

Milli-Q water was equilibrated at 4% O₂ and irradiated as indicated in [Table sup 1](#). Water samples were probed immediately after irradiation with Amplex Red assay kit (ThermoFisher). Fluorescence quantification was performed using Promega Glo-Max plate reader (Excitation: 520 nm Emission: 580–640 nm). G-value of hydrogen peroxide was calculated from the slope of plots of H₂O₂ concentrations as a function of the dose.

Zebrafish embryos experiments

AB Wild Type and transgenic Fli1a ZF embryos (to visualize the vascular tree) were irradiated 4 h to 4 h30 post-fertilization as indicated [Table sup 1](#). Survival and radiation-induced developmental alterations (n = 15 to 39 embryos) were investigated by measuring the body length 5 days post-fertilization as allowed by the Swiss ethics regulations. Temporal dynamics of radiation-induced cell death and proliferation were quantified on the whole body of ZF embryos (n = 5 embryos) using immunofluorescence and confocal microscopy analysis. Duplicate experiments were performed except for photon irradiation that was performed once.

Results

H₂O₂ yield is reduced at UHDR

We thought to use G-value of H₂O₂ in pure water as a surrogate for normal tissue sparing after UHDR irradiation. Therefore, the impact of dose and dose rate on H₂O₂ production and recombination when delivered with different type of beams was measured ([Fig. 1](#)). H₂O₂ yield increased linearly with the dose with all 3 beams. With 225 kV X-rays, the H₂O₂ yield was relatively higher and ranged between 3.8 to 4 molecules per 100 eV. For UHDR electron and proton beams H₂O₂ yield were globally lower, inversely proportional to the dose rate and did not vary linearly.

ZF embryos are sensitive to both the nature of the beam and dose rate

Dose and dose rate responses were investigated using a rapidly responding *in vivo* model with different beams. Dose responses were found for the 3 beams whereas dose rate responses were found for the electron but not for the proton beam. At isodose, two main patterns of response were found on ZF embryos: quasi-normal development/sparing effect and abnormal development/toxic effect. The FLASH sparing effect was found with electron at \geq 1400 Gy/s and proton at 0.1 and 1260 Gy/s with a minimal impact on embryo survival and growth 5 days post-fertilization ([Fig. 2a](#) and [b](#)). Toxicity was found with 225 kV photon and electron beam at conventional dose rate at respectively 0.037 Gy/s and 0.1 Gy/s. The photon beam was the most toxic with a 50% size reduction at 10 Gy and a high level of mortality (LD75 at 12 Gy) ([Fig. 2a](#)) whereas the lethal dose was never reached with the two other beams.

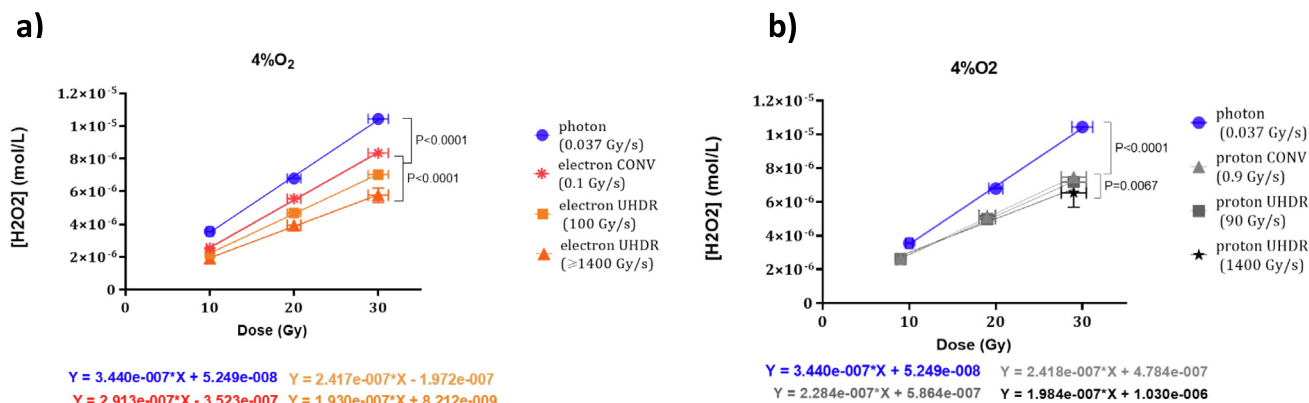
We speculated that developmental sparing effect induced by proton (conventional and UHDR) and electron UHDR could be

mediated by a decreased level of cell death and/or increased proliferation rate, to enable cellular mass recovery and preserve ZF development, whereas in photon and electron at 0.037 Gy/s and 0.1 Gy/s massive cell death could not be compensated. TUNEL and PhosphoH3 staining on full-body ZF embryos 24, 48 and 72 h post-irradiation were performed and analyzed by confocal microscopy and image reconstruction. Typical images of non-irradiated and ZF embryos 48 h post-irradiation at 10 Gy with the various beams (photon, electron and proton) are shown [Fig. 2c](#) and followed the two main patterns described above (normal vs abnormal). Non-irradiated embryos and embryos irradiated with proton beam (0.9 and 1260 Gy/s) and electron beam \geq 1400 Gy/s (1 pulse) showed quasi-normal developmental features. A dose dependent acute apoptotic peak was measured at 24 h in all irradiated groups, which is opposite to our hypothesis ([Figure sup 1 and 2](#)). However, with electron \geq 1400 Gy/s, it was compensated by a subsequent peak of proliferation 72 h post-irradiation. With proton (0.9 and 1260 Gy/s) and electron (\geq 1400 Gy/s), the apoptotic to proliferation ratio returned to the level of the control non-irradiated animals at 72 h post-irradiation, suggesting a full recovery ([Figure sup 1 and 2](#)). With photon and electron beam at conventional dose rate apoptosis rate occur early but was sustained over time, suggesting an ongoing radiation-induced cellular depletion process consistent with the severe morphological impact. Quantitative analysis was performed and is shown in [Figure sup 1 and 2](#), whereas [Figure sup 3](#) show the proportion of apoptosis vs proliferation in the different condition.

Discussion

Our results are the first systematic comparison of the radiolytic production of H₂O₂ and development of Zebrafish embryos after conventional and UHDR exposure with electron and proton beams. The temporal structure of the beam had no impact on H₂O₂ production but the mean dose rate did. A production of H₂O₂ \leq 2.33 molecule/100 eV also correlated with the preservation of ZF embryo development. Preserved developmental features also correlated with the onset of proliferation, presumably to compensate for apoptosis. Surprisingly, ZF embryos showed an exquisite sensitivity to the nature of the beam and dose rate. In our experiments, ZF embryos seemed resistant to proton irradiation, suggesting that this irradiation modality and/or beam configuration was intrinsically less toxic than low energy photon and electron beams in this specific model. These results point to the need for further systematic study; as such, an impact has not been reported *in vitro* or *in vivo*.

Understanding the physical parameters (*i. e.* mean dose rate vs instantaneous dose rate, time of exposure) able to trigger the FLASH effect is one important goal in the field of FLASH-RT research. Here, we took advantage of two beams (electron and proton) with intrinsic differences in temporal structure but able to operate at conventional and UHDR [[10,12](#)]. The maximal dose rate achievable with the proton beam was 1260 Gy/s delivered in a quasi-continuous manner whereas the electron beam was more flexible, able to deliver dose rates between 100 to 6.6.10⁶ Gy/s. For the latter, this flexibility was achieved by varying the number of pulses (see [Table sup 1](#)). In both cases dose uncertainty was around 4%. We compared the impact of conventional vs UHDR and electron vs proton beam on H₂O₂ production. H₂O₂ radiolytic yield was linear with the dose and inversely proportional to the dose rate. G° value of H₂O₂ after proton, electron and γ -rays at conventional dose rates are available and found to be similar [[14–18](#)], whereas direct measurement of H₂O₂ yields comparing conventional and UHDR irradiation has not been well investigated so far. Only one of our previous studies suggested that a decreased



c)

Oxygen level	4%O ₂						
Energy source	X-rays 225keV	Electrons 5.5MeV			Protons (transmission) 235MeV		
Mean Dose rate (Gy/s)	0.037	0.1	100	≥1400	0.9	90	1260
G(H ₂ O ₂) (molecules/100eV)	3.32±0.07	2.81±0.03	2.33 ±0.03	1.86±0.05	2.33±0.05	2.20±0.04	1.92±0.15
Decrease (%) in UHDR G(H ₂ O ₂) from conventional G(H ₂ O ₂)	N.A	N.A	17	34	N.A	6	18

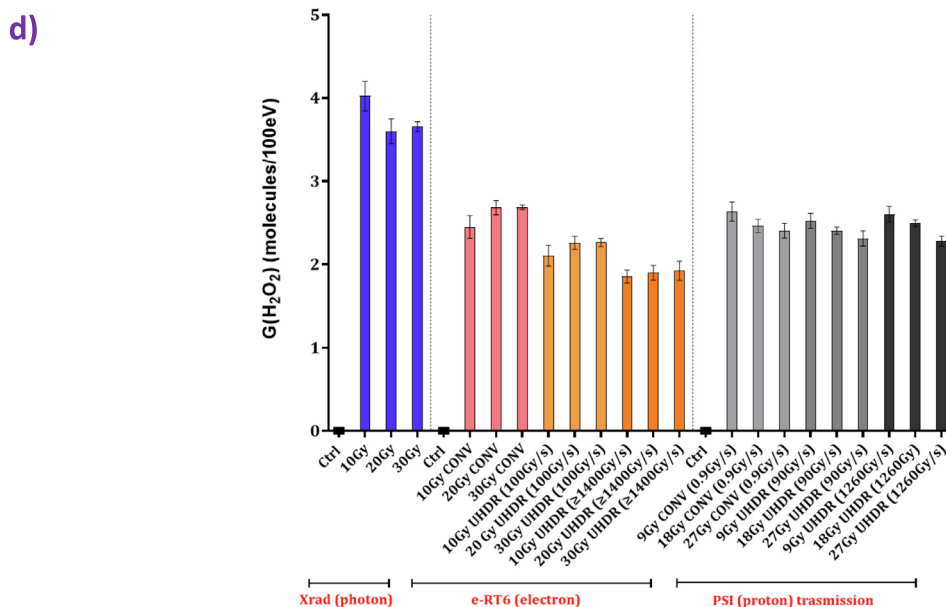


Fig. 1. Dose and dose rate impact on water radiolysis in physiological O₂ conditions (4% O₂) after exposure to photon, electron and proton beam a) [H₂O₂] concentrations vs the irradiated dose obtained after water exposure to X-rays, electrons (CONV and UHDR) b) Similar plot obtained with proton beam (conventional and UHDR). Slopes were assessed by t-test and were significantly different as follow: photons vs electrons (CONV&UHDR): P < 0.0001; CONV electrons vs UHDR electrons: P < 0.001 and CONV protons vs UHDR protons: P = 0.0067 c) Summary table d) Radiolytic yield of H₂O₂ obtained from the previous figure by dividing [H₂O₂] over the dose. Uncertainties on the dose for electron and proton irradiations were 4% and 10% respectively from the prescribed dose. Results are from duplicate experiments for X-rays and protons irradiation and triplicate experiments for electrons irradiations.

production of H₂O₂ after exposure to UHDR electron (>100 Gy/s) could trigger normal tissue sparing in ZF embryos' and mouse [1,19]. Here, we calculated G values of H₂O₂ produced by photon, electron and proton beams and could ranked them from the highest production to the lowest: CONVphoton > CONVelectron > CON

Vproton > UHDRproton > UHDRelectron. We also found that 2.33 molecules of H₂O₂/100 eV or less correlated with the preservation of ZF embryo development. Whether this value can be considered as a threshold and/or a surrogate marker of normal tissue protection remains to be established.

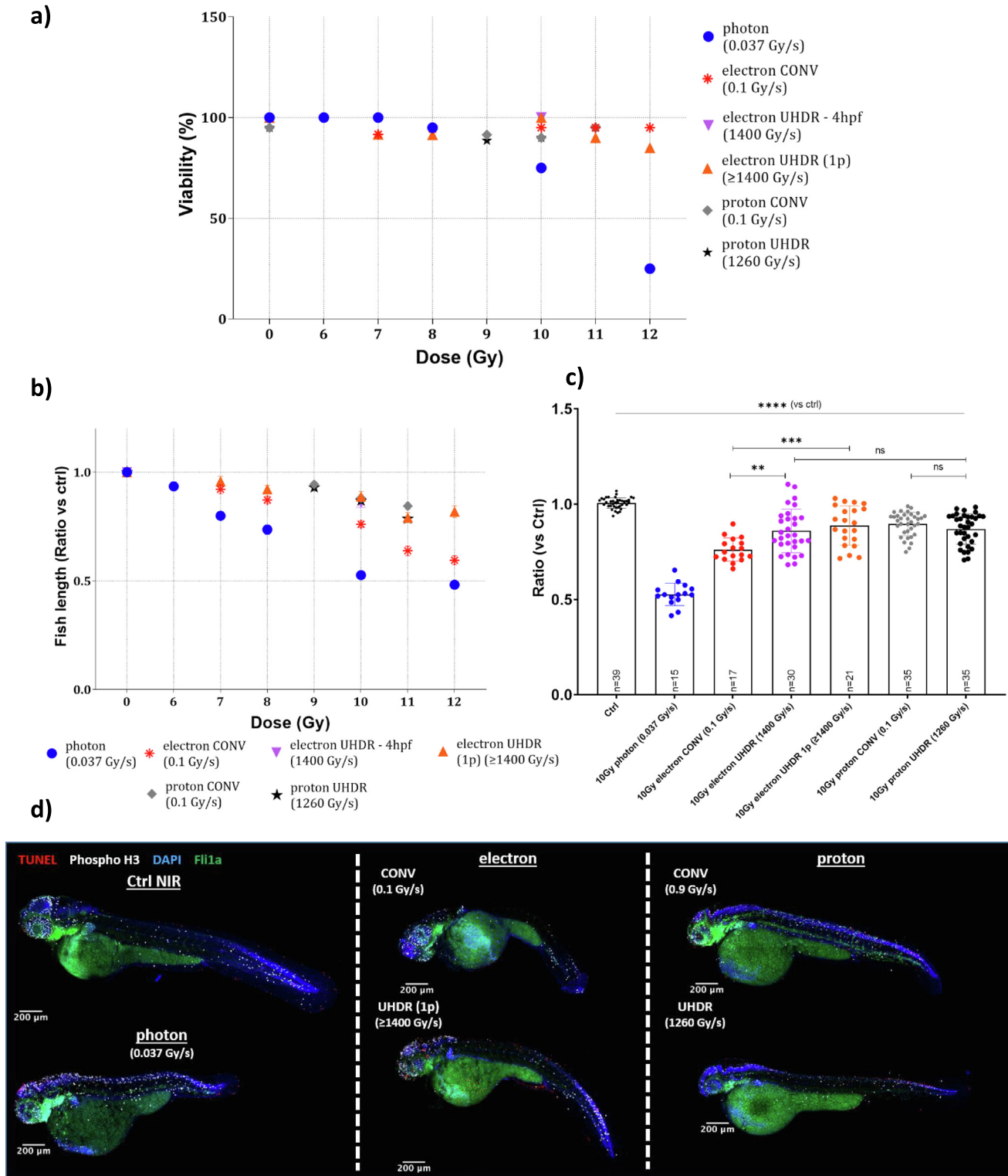


Fig. 2. Dose and dose rate impact on zebrafish embryos exposed to photon, electron and proton beams 4–4.30 h post-fertilization. a) Embryonic survival b) Fish length assess developmental retardation induced by irradiation at 5 days post-fertilization. (Embryos n = 20 per each dose, results are the average of two experiments) c) Fish length in bargraph at 10 Gy d) Comparison of radiation induced cell death and apoptosis response in ZF embryos (Fli1a) triggered by TUNEL assay and Phospho H3 staining after irradiation with 10 Gy (48 h post RT) using photon, electron and proton beam at different dose rates. Images were acquired over the embryo total body with confocal microscopy (10x); Red: TUNEL, White: Phospho H3, Blue: DAPI and Green: Fli1a. (Embryos n = 5 per each dose).

The ZF embryo is relatively new model in the field of radiation biology. It has several distinct advantages including the fact that it is a fully integrated in vivo model and it's a short-term model with a fast biological response (5 days). These embryos also have other

interesting characteristics such as a very rapid shift in their radiation resistance profile according to developmental status (DL100 ranging from 15 to 50 Gy within the first 24 h of their development) [20]. To investigate the impact of clinically relevant doses

(in the range of 10 Gy), we chose to work at 4–4.30 hpf. At this early stage of their development, ZF embryos can be considered to be stem cell-like, behaving like acute responding tissues. A lower magnitude of the FLASH sparing effect (about 10%) found here is similar to what has been reported in other early responding tissues such as the gut of mice [21,22]. Interestingly in this study, ZF embryos appear to be more sensitive to the nature of the beam and/or dose rate variation than mice for reasons that remain to be determined. Other nuances of the ZF model exist, where a past report showed no FLASH sparing effect with proton beams at 100 Gy/s [23]. Conversely, normal tissue sparing by proton FLASH beams (>70 Gy/s) has been consistently reported by many groups working with various murine models (gut, skin, brain) [3,5–7]. Our present results are consistent and extend Beyreuther's previous results showing no FLASH effect between 0.1 Gy/s and 1260 Gy/s with proton beams. Interestingly, the apparent lack of the FLASH effect was not related to enhanced damage, but rather to a preservation of ZF development after proton irradiation at either dose rate. When compared with photon and electron beams at conventional dose rates, a DMF of 1.6 and 1.1 was found respectively, whereas at UHDR ZF embryo development was similar with proton and electron beam. Recently, Beyreuther's group suggested that hypoxia was required in ZF embryos to trigger the FLASH sparing effect in response to UHDR with VHEE [24], a factor that was not found to be required in our previous experiments with 5.5 MeV. [19,25] The current investigation did not evaluate this, since we chose to work at physiologically relevant normoxic conditions. Here, the molecular pattern associated with the preservation of ZF embryo development was investigated and found to be beam and dose rate independent for protons but not for electrons. In fact, full recovery of the ZF embryo observed after UHDR electron and proton irradiation was associated with an early apoptotic peak followed by high levels of compensatory proliferation. This was not found however, after conventional dose rate photon and electron exposures, where sustained apoptosis was not compensated by proliferation.

Conclusions

In summary, our observations point to an unexpected but significant protective advantage of proton irradiation on ZF embryos that is coincident with a lower production of H₂O₂. The translation of these findings to higher mammals is unclear but suggests that investigations to systematically characterize the impact of dose rate modulation using protons, photons and electrons response is clearly needed. Our findings also suggest that H₂O₂ ≤ 2.33 molecule/100 eV might serve as marker of the FLASH sparing effect and identifies transient vs sustained apoptosis has a possible switch leading to normal vs abnormal development of ZF embryos.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The proton studies were funded by a research grant from Varian, a Healthineers company (Palo Alto, CA, USA) (to MCV, SP and TL).]

Acknowledgements

HK and electron studies were funded by MAGIC-FNS CRS I15_186369 (to MCV) and proton studies were funded by a research grant from Varian, a Healthineers company (Palo Alto, CA, USA) (to MCV, SP and TL), VG was funded by NIH program project grant PO1CA244091 (to MCV). We would like to thank Drs P Froidevaux

and C Bailat for their critical contribution; Paula Barrera-Gomez and the PTZ from UNIL for ZF breeding and well as Dr A Benechet from IVIF/UNIL/CHUV and Dr F Morgenthaler from CIF for help in imaging procedures.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.radonc.2022.07.011>.

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