Single low-dose ionizing radiation induces genotoxicity in adult zebrafish and its non-irradiated progeny

J. Lemos[a,b], T. Neuparth[c], M. Trigo[d], P. Costa[b], D. Vieira[b], L. Cunha[e], F. Ponte[f], P.S. Costa[f], L.F. Metello[b,e], A.P. Carvalho[c,d]

[a] ICBAS – Institute of Biomedical Sciences, University of Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[b] Nuclear Medicine Dept., High Institute for Allied Health Technologies of Porto - Polytechnic Institute of Porto (ESTSP.IPP), Rua Valente Perfeito 322, 4400 - 330 Vila Nova de Gaia, Portugal
[c] CIIMAR/CIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal
[d] Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal
[e] IsoPor SA, PO box 4028, 4445, Ermesinde, Portugal
[f] Radiotherapy Dept., Júlio Teixeira SA, Rua Arquitecto Cassiano Barbosa 6, F, Sala 26, 4100-009 Porto, Portugal

Corresponding author:
António Paulo Carvalho
Department of Biology, Faculty of Sciences, University of Porto
Rua do Campo Alegre, 4169-007 Porto, Portugal
E-mail: apcarval@fc.up.pt / Phone: +351 220 402 761 / Fax : +351 220 402 709

Abstract

This study investigated to what extent a single exposure to low doses of ionizing radiation can induce genotoxic damage in irradiated adult zebrafish (*Danio rerio*) and its non-irradiated F1 progeny. Four groups of adult zebrafish were irradiated with a single dose of X-rays at 0 (control), 100, 500 and 1000 mGy, respectively, and couples of each group were allowed to reproduce following irradiation. Blood of parental fish and whole-body offspring were analysed by the comet assay for detection of DNA damage. The level of DNA damage in irradiated parental fish increased in a radiation dose-dependent manner at day 1 post-irradiation, but returned to the control level thereafter. The level of DNA damage in the progeny was directly correlated with the parental irradiation dose. Results highlight the genotoxic risk of a single exposure to low-dose ionizing radiation in irradiated individuals and also in its non-irradiated progeny.

Keywords: Zebrafish, ionizing radiation, genotoxicity, comet assay

Introduction

Living organisms are chronically exposed to low doses of ionizing radiation in their environment. Natural sources (e.g. cosmic rays and radioactive substances in the earth's crust) are by far the major cause of this background radiation, but increasingly additional contributions have been given by anthropogenic sources over the past century (UNSCEAR 2010). Anthropogenic sources of radiation mainly include nuclear weapons use and testing, nuclear power production and accidents in nuclear power plants, as well as the use of radiation in medical procedures. The medical use of radiation has become the major man-made source of ionizing radiation exposure to humans, and is a growing concern for professionals of medical radiology due to occupational exposure (UNSCEAR 2010).

The DNA molecule is the primary target of ionizing radiation within the cell, and biological effects of radiation originate mostly from DNA damage. Ionizing radiation can induce DNA damage by changing the molecule chemical structure either directly or indirectly via radiation-generated reactive radicals (Harrison 2013). A multiplicity of radiation-induced DNA damages has been identified, including single-strand and double-strand breaks (Harrison 2013). If these damages are not efficiently repaired by naturally occurring DNA repair mechanisms, un- or mis-repaired DNA can lead to chromosomal abnormalities, gene mutations, cancer, and cell death. As a precautionary rule, it has been generally accepted that there is no safe dose of radiation – any amount increases the risk of damage (Mothersill and Seymour 2011; Duport et al. 2012). A linear no-threshold model has therefore been assumed for low-dose radiation, stating that the risk of damage is directly proportional to exposure dose.

Considering the carcinogenic potential of low-dose ionizing radiation, several studies have focused on the effects of radiation at low doses in humans (Sari-Minodier et al. 2007; Ropolo et al. 2012; Saberi et al. 2013; Tug et al. 2013; Han et al. 2014) or have estimated the increased risk of cancer associated to such radiations (review in Prasad 2012). In general, these are epidemiological studies that compare retrospectively the incidence of a given effect in a selected group of previously exposed individuals (e.g. hospital radiology workers, patients irradiated for medical purposes, survivors from nuclear accidents) and a similar group of unexposed individuals. Although epidemiological studies in humans provide relevant information about health risks associated to low-dose ionizing radiation, they are subject to important constraints in terms of statistical power, uncontrolled variables,

75 exposure misclassification, and selection bias (Duport et al. 2012). Experimental studies in
76 cell cultures or laboratory animals, allowing working with populations with low individual
77 variability and testing a wide range of accurate doses of radiation under strict control of all
78 covariates, are a valuable alternative to assess the biological effects of radiation. On the other
79 hand, since *in vitro* systems can respond differently to radiation comparing to *in vivo*
80 systems (Jarvis and Knowles 2003; Bladen et al. 2007; Duport et al. 2012), experiments with
81 laboratory animals can give us more precise insight into the effects of radiation and their
82 underlying mechanisms.
83 Mammals (small rodents and dogs) are the most frequently used animals in experimental
84 radiobiology (Duport et al. 2012). The zebrafish (*Danio rerio*) has become widely used as an
85 *in vivo* model in many areas of biomedical research, but its utilization in radiobiology is still
86 scarce and almost restricted to the embryonic stage. However, a number of favourable
87 features, such as short generation time, easy reproduction and high fecundity, make this
88 small teleost fish particularly suitable for studies on long-term and transgenerational effects
89 of radiation. Moreover, since zebrafish and human genomes share a substantial degree of
90 homology, including with regard to most DNA repair-related genes (Geiger et al. 2006),
91 radiation studies in zebrafish can provide valuable information on radiation-induced human
92 cancers. Finally, radiation studies in zebrafish, used as a model, can also be useful from an
93 ecotoxicological point of view. In fact, aquatic ecosystems are prone to accidental or
94 intentional contamination by radionuclides that undergo radioactive decay, resulting in the
95 emission of ionizing radiation, whose impact on aquatic organisms must be evaluated
96 (Matranga et al. 2010; Reinardy et al. 2011; Simon et al. 2011; Anbumani and Mohankumar
97 2012; Praveen Kumar et al. 2014; Saiyad Musthafa et al. 2014).
98 Experimental studies on the effects of low-dose ionizing radiation (up to 1 Gy) in living
99 organisms have focused mostly on chronic exposure (from few hours to several months) and
100 little attention has been paid to effects of single irradiation. In the present study we used the
101 zebrafish as a biological model aiming at (1) investigating to what extent a single exposure
102 to low doses of ionizing radiation, within the low-dose range for medical practice (≤1Gy),
103 can induce DNA damage in sexually mature individuals, and (2) evaluating the possibility of
104 transmission of damage to the non-irradiated F1 progeny.
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106
107 **Materials and Methods**
108
109 A group of sexually mature one year-old zebrafish purchased from a commercial
110 supplier was kept in aquaria at water temperature of 25±2°C and photoperiod of 14h
111 light/10h dark, fed ad libitum twice a day with commercial flaked food, for about two
112 months prior to irradiation. After that time, fish were sexed and distributed by four groups of
113 thirty six individuals, eighteen of each sex. Three of these groups were externally irradiated
114 with a single emission of X-ray at distinct doses: respectively 100 mGy (8 MU for 0.02
115 min), 500 mGy (42 MU for 0.1 minutes) and 1000 mGy (using 83 MU for 0.2 minutes); the
116 remaining group was subject to the same handling as the other groups excepting that was not
117 irradiated, serving as a control. The irradiation was performed using a Varian 6 MV linear
118 accelerator, with fish placed inside a container with a homogeneous field of 200 mL of water
119 (1.5 cm depth), at 1 m away from the beam source. An ionization chamber (0.6 cc PTW TM
120 30013) was used to confirm the desired doses. After irradiation, from each group, two
121 couples of fish were housed in a separate appropriate cage for reproduction and the
122 remaining fish were placed in an independent aquarium. A sample of five males and five
123 females was taken from each aquarium/group at days 1, 4 and 7 post-irradiation to evaluate
124 genotoxicity induced by radiation. Fertilized eggs of the first spawning from breeding

125 couples of each group were collected, incubated until hatching, and five pools of five newly-
126 hatched larvae (3 days post-fertilization) per group were sampled to evaluate genotoxicity in
127 the progeny of irradiated parents.
128 Genotoxicity was assessed by measuring the level of DNA damage (DNA strand breaks)
129 through the alkaline comet (single-cell gel electrophoresis) assay, performed according to
130 Singh et al. (1988) and as previously described by Neuparth et al. (2013). In adult fish the
131 effects were assessed in peripheral blood of each individual (five males + five females per
132 group and per sampling time): blood was collected just above the lateral line system with a
133 syringe previously washed with 0.1 M EDTA to prevent clotting, and blood cell suspensions
134 were obtained by diluting (1:100) blood aliquots in cold homogenizing buffer (75 mM NaCl,
135 24 mM Na2EDTA, pH 7.5). In larvae the effects were assessed in whole body homogenates
136 of pooled individuals (five pools per group): pools of five larvae were macerated in cold
137 homogenizing buffer (75 mM NaCl, 24 mM Na2EDTA, pH 7.5), and homogenates were
138 obtained by filtering through a 60 µm filter. Analysis was run immediately after blood
139 collection (for adults) or whole body homogenization (for larvae) to ensure maximum cell
140 viability. For that, 10 µl of blood cell suspensions or larval homogenates were diluted in
141 liquid (37ºC) 1% w/v low-melting point agarose and placed (2×75 µl) on microscopy slides
142 previously coated with high melting point agarose. After the gel had set, the slides were
143 placed into a cold lysing solution for 1h (2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, pH
144 10, 1% Triton X-100, 10% DMSO). Slides were then placed in cold alkaline electrophoresis
145 solution (0.1 mM Na2-EDTA, 0.3 M NaOH, pH 13), for 40 min. Electrophoresis was run for
146 30 min at 25 V using a horizontal gel electrophoresis tank. Slides were afterwards
147 neutralized in cold Tris–HCl buffer (pH 7.5), and then dehydrated with ice-cold absolute
148 methanol to be preserved until analysis. Before the examination, the slides were rehydrated
149 and then stained with 0.02 mg/L ethidium bromide. A total of 100 randomly chosen cells
150 were scored per slide under a fluorescence microscope and the comets were analysed using
151 the software Comet Score 1.5 (TriTek Corp., Summerduck, USA). The percentage DNA in
152 the tail, one of the most consensual and reliable comet metrics, was employed as a direct
153 measure of DNA-strand breakage (Lee and Steinert 2003; Kumaravel and Jha 2006).
154 Data obtained with adult fish were firstly analysed by factorial (three-way) ANOVA, at
155 the significance level of 0.01, to find if there was an interaction effect of factors (radiation
156 dose, day post-irradiation, sex) on the magnitude of DNA damage. When a significant
157 interaction occurred, one-way ANOVA was performed to identify the effect of one factor for
158 each level of the remaining factors. In the case of larvae, since only one factor was studied
159 (the radiation dose of parental exposure), data were analysed by one-way ANOVA. In both
160 cases, when significant differences were detected by one-way ANOVA at the significance
161 level of 0.01, means were compared by the Tukey multiple-range test. Analyses were
162 performed using the software IBM SPSS Statistics version 22.
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164
165 **Results and Discussion**
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167 The radiation dose and the day post-irradiation had a significant overall effect on the level
168 of DNA damage detected in irradiated adult zebrafish, contrarily to the sex of fish (Table 1).
169 Moreover, a significant interaction effect of radiation dose and day post-irradiation was also
170 observed (Table 1), meaning that the dose-response relationship was not the same in all days
171 post-irradiation. Therefore, independent one-way ANOVAs were performed to compare the
172 effect of dose in each day post-irradiation. For this, since both sexes responded identically,
173 data obtained for males and females exposed to the same radiation doses in the same days
174 post-irradiation were analysed together (5 females + 5 males, n=10).

175 **Table 1** Results of the factorial ANOVA to assess the effect of radiation dose, time after
176 irradiation and gender on DNA damage of irradiated adult zebrafish

Source of variation	*df*	*F*	*Significance*
Radiation dose	3, 96	4.56	< 0.01
Day post-irradiation	2, 96	10.87	< 0.01
Sex	1, 96	6.17	> 0.01
Radiation dose × Sex	3, 96	1.32	> 0.01
Day post-irradiation × Radiation dose	6, 96	4.12	< 0.01
Sex × Day post-irradiation	2, 96	3.28	> 0.01
Radiation dose × Day post-irradiation × Sex	6, 96	1.85	> 0.01

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179 The level of DNA damage induced by radiation in adult zebrafish was positively dose-
180 dependent at day 1 post-irradiation, and significantly higher in fish exposed to the highest
181 dose than in control fish (Fig. 1). Relatively to day 1, at days 4 and 7 post-irradiation the
182 level of DNA damage decreased in all irradiated groups and returned to the control level
183 (Fig. 1).
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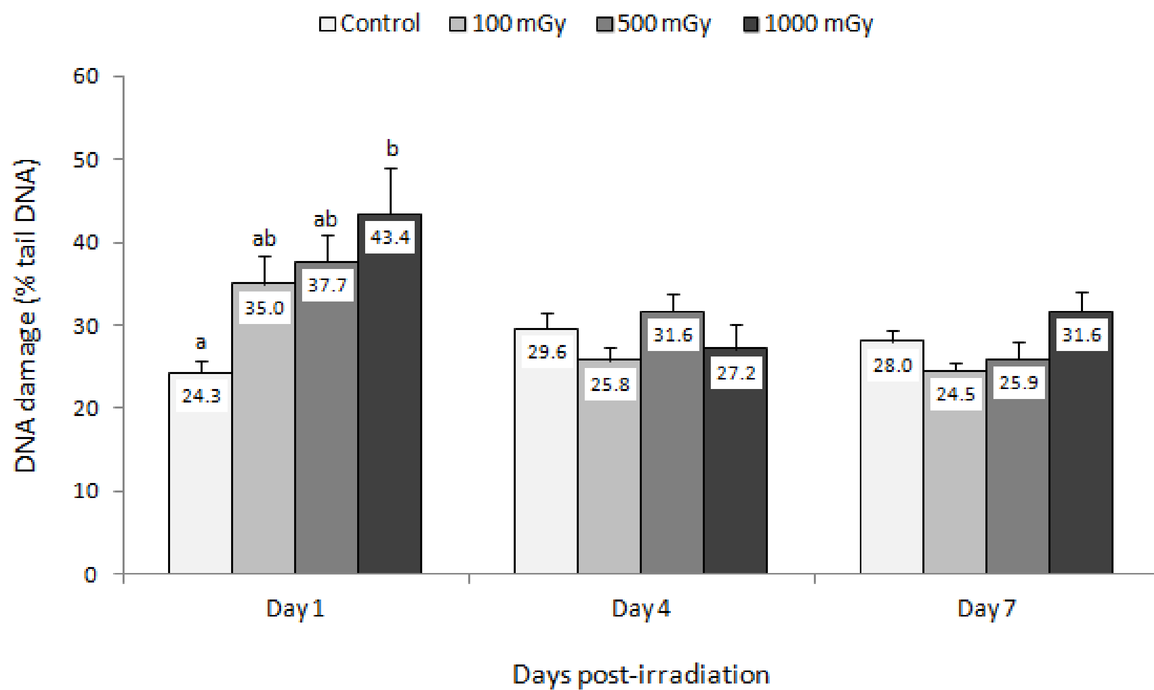

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206 **Fig. 1** Level of DNA damage in adult zebrafish at days 1, 4 and 7 post-exposure to different
207 doses of ionizing radiation. Values are mean of n=10; error bars indicate the standard error
208 of mean. For each day, column values with different letters are significantly different
209 (*P*<0.01). Mean values of the control group at days 1, 4 and 7 are not significantly different.
210
211
212 It is well established that DNA strand breakage is a major early biological effect of
213 ionizing radiation. Exposure of diploid mammalian cells to 1 Gy of radiation can generate
214 about 1000 single- and 30 double-strand breaks per cell (Olive 2009). The alkaline comet

215 assay is a rapid and sensitive technique to detect both kinds of DNA strand breaks (Collins et
216 al. 2008; Collins 2015), and its usefulness in assessing DNA damage resulting from
217 exposure to ionizing radiation has been well recognized (Kumaravel and Jha 2006; Collins et
218 al. 2008; Olive 2009). Since the maintenance of DNA integrity is of chief importance, all
219 living organisms developed efficient mechanisms for repairing DNA damage induced by
220 genotoxicants. However, some DNA strand breaks can persist, depending, among other
221 factors, on the repairing ability of the organism, on the dose of genotoxicant, and on the
222 extent of exposure (Everaarts 1995; Shugart 2000). The return of DNA damage to the
223 control level that we observed at day 4 post-irradiation can thus suggest the repairing of
224 DNA strand breaks induced by radiation. Similarly, studies in tilapia (*Oreochromis*
225 *mossambicus*) and rohu (*Labeo rohita*) showed a time-dependent decrease of DNA damage
226 detected by the comet assay in blood cells (and other tissues) after cessation of exposure to
227 different toxicants, which was interpreted as the result of the DNA repairing activity carried
228 out by fish (Banu et al. 2001; Ahmed et al. 2011; Mohanty et al. 2011). Previous findings,
229 indicating high DNA repair capacity in zebrafish embryos (Sussman 2007) and rapid
230 activation of genes associated with DNA repair, following induction of damage, in zebrafish
231 larvae (Reinardy et al. 2013), seem to support our results.
232 The induction of genotoxic effects by ionizing radiation has been confirmed in different
233 fish species, such as medaka (*Oryzias latipes*) (Kubota et al. 1995; Grygoryev et al. 2013)
234 and Indian carp (*Catla catla*) (Anbumani and Mohankumar 2012, 2015). However, studies in
235 these species have tested protracted or chronic exposure to radiation, in many cases at very
236 high doses, instead of a single exposure to a low-dose radiation as in our study. In zebrafish,
237 evaluation of genotoxic effects of ionizing radiation has been focused on early stages of
238 development, i.e., embryos and larvae (Jarvis and Knowles 2003; Simon et al. 2011;
239 Gagnaire et al. 2015), exposed to gamma-rays from very low to low doses (0.4-1000 mGy),
240 for a variable period of time (1 h to 20 days). DNA damage in early life stages of zebrafish
241 were found at accumulated doses as low as 1.2 mGy (1.2 mGy/h for 1h) (Jarvis and Knowles
242 2003), which represents an accumulated dose of about 100 times lower than the lowest
243 single dose we tested in adult zebrafish. Although the exposure time may have influence
244 when comparing these results, it is expectable that fish at early developmental stages are
245 much more radiosensitive than adult fish. Actually, initial developmental stages are
246 characterized by exponential growth and ongoing organ differentiation, with high rate of cell
247 proliferation that renders DNA more vulnerable to radiation and mistakes of repair
248 mechanisms.
249 In fish that were held to reproduce, spawning and subsequent fertilization occurred at the
250 first day post-irradiation in the non-irradiated control group and at the third day post-
251 irradiation in all irradiated groups. Since newly-hatched larvae were sampled three days after
252 fertilization, this means that the level of DNA damage in larvae was assessed six days after
253 parental irradiation. As spawning in zebrafish can be quite variable and no statistical analysis
254 was performed due to lack of replicates, we cannot confirm if the spawning delay in
255 irradiated groups was related to radiation exposure.
256 The level of DNA damage in the non-irradiated F1 progeny (newly-hatched larvae) was
257 directly correlated with the radiation dose of parental exposure (Fig. 2), and reflected the
258 initial level of DNA damage of the respective parents. Statistically, the level of DNA
259 damage was significantly higher in offspring from parents exposed to the highest radiation
260 dose than in the others (Fig. 2). Damages may have been transferred to the progeny through
261 parental damaged germ cells, most likely through damaged sperm. Indeed, at least in
262 mammals, there is evidence that post-meiotic male germ cells lose the ability to repair their
263 DNA, contrarily to the oocyte (Harrouk et al. 2000). Thus, DNA lesions carried by the
264 parental sperm may induce damage in the zygotes and in developing embryos, causing

265 genomic instability that can persist through generations (Adiga et al., 2010). It has been
266 suggested a delay in the activation of pathways inducing the genomic instability mediated by
267 parental damages, retarding for a few days after fertilization the onset of the genotoxic
268 response in the progeny (Adiga et al. 2010). Once this response is triggered, the intensive
269 cell proliferation and differentiation that characterize developing embryos will lead to a high
270 propagation of damaged cells. Considering that embryos also possess an efficient DNA
271 repair capacity, this helps to explain the persistence of the relative high level of DNA
272 damage in newly-hatched larvae, six days after parental irradiation, when damages in parents
273 had already returned to the baseline. Comparable results were found in a study in mice,
274 where comet assay was used to evaluate DNA damage in the sperm of irradiated males and
275 its non-irradiated progeny (Adiga et al. 2010). In that study, the level of DNA damage in the
276 sperm of the first-generation offspring also reflected the level of DNA damage in the
277 parental sperm that, in turn, was radiation dose-dependent. Moreover, it was proved that the
278 genetic damage was also transmitted to the somatic line of the progeny (assessed by
279 increased percentage of micronuclei in fetal liver cells), following the same trend observed
280 in the germ line. In our study, since homogenates of whole-larvae were used in the comet
281 assay, damage found in offspring cannot be assigned to any particular cell type. Our results
282 in zebrafish and those of Adiga et al. (2010) in mice support previous findings in medaka
283 fish on the occurrence of mutations in the progeny of irradiated parents (Kubota et al. 1995;
284 Shimada and Shima 2001, 2004). The transmission of genetic damages to future generations
285 is responsible for transgenerational genomic instability, an important non-targeted, delayed
286 effect of ionizing radiation (Barber and Dubrova 2006; Choi and Yu 2015).
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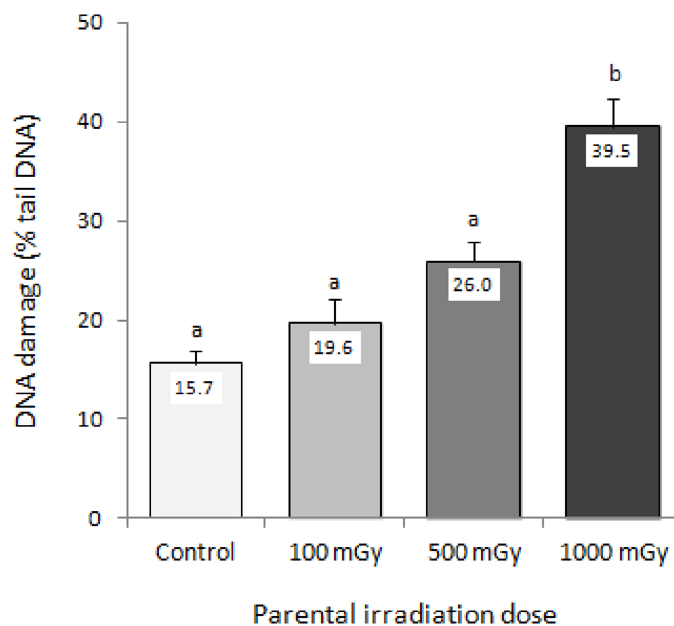

307 **Fig. 2** Level of DNA damage in the unexposed progeny (newly-hatched larvae) of adult
308 zebrafish exposed to different doses of ionizing radiation. Values are mean of n=5; error bars
309 indicate the standard error of mean. Column values with different letters are significantly
310 different (*P*<0.01)
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312
313 Overall results highlight the genotoxic risk of a single exposure to low-dose ionizing
314 radiation in irradiated zebrafish adults and also in its non-irradiated F1 progeny. Moreover,

315 this work confirms the potential of zebrafish as an *in vivo* model in experimental
316 radiobiology. Considering the present findings, further studies should be undertaken to
317 provide insight into the transgenerational effects of ionizing radiation.
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319

320 Acknowledgments

321

322 We are grateful to the staff of the bioterium of aquatic organisms (BOGA) from CIIMAR
323 and to Júlio Teixeira SA – Radiotherapy Department for their help in zebrafish maintenance
324 and zebrafish irradiation, respectively.
325
326

327 Ethical approval

328

329 All applicable international, national, and/or institutional guidelines for the care and use
330 of animals were followed.
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332

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