Effects of tritiated water on locomotion of zebrafish larvae: a new insight in tritium toxic effects on a vertebrate model species

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[1] Institut de Radioprotection et de Sûreté Nucléaire (IRSN), PSE-ENV/SRTE/LECO, Cadarache, 13115, Saint-Paul-lez-Durance, France

[2] Institut de Radioprotection et de Sûreté Nucléaire (IRSN), PSE-SANTE\SDOS\LMDN, Cadarache, 13115, Saint-Paul-lez-Durance, France

Corresponding authors:

arcanjo.caroline@hotmail.fr and beatrice.gagnaire@irsn.fr

Abstract

Tritium (^{3}H), a radioactive isotope of hydrogen, is ubiquitously present in the environment. In a previous study, we highlighted a mis-regulation of genes involved in muscle contraction, eye transparency and response to DNA damages after exposure of zebrafish embryo-larvae from 3 hpf to 96 hpf at 0.4 and 4 mGy/h of tritiated water (HTO). The present study aimed to link this gene mis-regulation to responses observed at higher biological levels. Zebrafish embryo-larvae were exposed to 0.4 and 4 mGy/h. Analyses on spontaneous tail movement, locomotor activity and heart rate were performed. Histological sections of eyes were made to evaluate the impact of HTO on eye transparency and whole embryo immunostainings were realized to assess DNA double strand breaks repair using gamma-H2AX foci. We found a decrease of basal velocity as well as a decrease of response in 96 hpf larvae exposed at 0.4 mGy/h after a tactile stimulus as compared to controls. Histological sections of larvae eyes performed after the exposure to 4 mGy/h did not show obvious differences in lens transparency or retinal development between contaminated and

control organisms. Gamma-H2AX foci detection revealed no differences in the number of gamma-H2AX foci between contaminated organisms and controls, for both dose rates. Overall, results highlighted more detrimental effects of HTO exposure on locomotor behavior in 96 hpf larvae exposed at the lowest dose rate. Those results could be linked to mis-regulation of gene involved in muscle contraction found in a previous study at the same dose rate. It appears that not all effects found at the molecular scale were confirmed using higher biological scales. These results could be due to a delay between gene expression modulation and the onset of physiological disruption or homeostatic mechanisms to deal with tritium effects. However, crossing data from different scales highlighted new pathways to explore, i.e. neurotoxic pathways, for better understanding HTO effects on organisms.

Keywords

Tritiated water; Zebrafish embryo-larvae; locomotor-related behavior ; heart-beats ; eye histology; DNA double strand breaks

1. Introduction

Organisms are largely exposed to radionuclides originating from natural or anthropic sources. Among them, tritium (^{3}H) can be found in the environment coming from both sources. As a radioactive isotope of hydrogen, tritium enters in the water cycle and therefore is present in all environmental compartments (Adam-Guillermin et al., 2010). The decay of tritium, following a half-time of 12.32 years, emits a beta particle of low energy (mean = 5.7 keV) and low penetration range (mean = 0.56 µm in water) (HPA, 2007). This last property implies that the energy deposition in tissues is very localized and gives to tritium a high ionization capacity that can be considered negligible for external exposure (Mathur-De Vre and Binet, 1984). However, if tritium is integrated in organism tissues, the effects could be more detrimental than external exposure.

The tritium activity background, i.e. without nuclear facilities influences, in southern France rivers ranges from 0.12 to 0.86 Bq/L (Ducros et al., 2018). In marine environment, the background of tritium activities ranges from 0.02 to 0.15 Bq/L (Eyrolle-Boyer, 2017). In

France, tritium is mainly discharged in the environment by nuclear power plants and nuclear reprocessing plants in normal operations in the form of tritiated water (HTO) or tritiated hydrogen (HT). The average activity measured in nuclear power plants liquid effluents in normal operations is of $2x10^6$ Bq/L making tritium one of the most discharged radionuclides in terms of activity compared to other radionuclides (Adam-Guillermin et al., 2010). However due to the tritium properties of rapid dilution in the water cycle, activities measured in rivers downstream of the nuclear facilities range from 1 to 65 Bq/L (Eyrolle-Boyer, 2017). In the English Channel, downstream of the nuclear facilities tritium activity is about 10 Bq/L and gradually decrease along the coast (Eyrolle-Boyer, 2017). In the future, the development of new nuclear facilities, such as European Pressurized Reactor (EPR) or International Thermonuclear Experimental Reactor (ITER), is expected to increase tritium discharge in environment. That is why, understanding the risks of tritium on aquatic organisms is of growing concern in the scientific community.

The effects of tritiated water exposure were studied over a large range of species, from mammals through the effects on reproduction in mice or rats (Cahill and Yuile, 1970; Laskey et al., 1973; Lowry Dobson and Cooper, 1974) and human cells to assess DNA damages (Gonen et al., 2014) to aquatic invertebrates or vertebrates (Adam-Guillermin et al., 2012). For those organisms, studies ranged from *in vitro* (Stuart et al., 2016) to *in situ* (Gagnaire et al., 2017) through laboratory studies mainly focusing on developmental and reproductive effects (Adam-Guillermin et al., 2012). Despite the wide range of species in which effects of HTO exposure were studied, there is still a lack of knowledge of tritium modes of action at the molecular scale, the transgenerational effects, the adaptation or the behavior of organisms (Adam-Guillermin et al., 2012, 2010).

The present work is part of a project which aims to identify the modes of action of tritiated water. Explaining that activities chosen for exposures are widely above those found in natural environment, under nuclear facilities influence or not. To reach this objective, an aquatic vertebrate model was used, the zebrafish, *Danio rerio*. The embryo larval stages were chosen, as early developmental stages are supposed to be more sensitive to pollutant and as the effects of pollutants on this early stage could induce detrimental effects during the rest of the fish development (Fraysse et al., 2006). Furthermore, these early stages are recommended in OECD guidelines for the testing of chemicals (OECD, 2013). Zebrafish is a

very convenient laboratory model especially when the use of the embryo-larvae stages is required, as in this project. Indeed, adults can spawn hundreds of transparent eggs in a single spawning (Spence et al., 2008). The genome of this species is fully sequenced, and a wide variety of mutants are now available (Scholz et al., 2008), which is useful when focusing on modes of action. Furthermore, zebrafish is an emerging model for studying behavior and many tools are now available to assess plenty of behavioral parameters in adult and embryo-larvae of zebrafish (Ahmad et al., 2012). In this project, a previous study was conducted and focused on the effects of HTO exposure on zebrafish early life stages at the molecular scale using a transcriptomic analysis by mRNAseq (Arcanjo et al., 2018). This study revealed an effect of HTO exposure on the expression of genes involved in muscle contraction, eye transparency, circadian clock, response to reactive oxygen species and repair of DNA damages. The aim of the present study was to complete the results of this previous work by investigating if the effects observed at the molecular scale were confirmed at higher biological scales including tissue and individual, more relevant from an ecological point of view. To reach this objective, 3 hpf (hours post-fertilization) zebrafish eggs were exposed to the same dose rates that were previously used (i.e. 0.4 and 4 mGy/h). Both of these dose rates corresponded to a band of dose rates within which there is some chance of observing some deleterious effects on young fish (e.g. reduction in resistance to infection and reproductive success) (ICRP, 2012). In term of activity concentrations, these two doses rates corresponded to tritiated water activities of $1.22x10^5$ and $1.22x10^6$ Bq/mL, respectively. As the first part of the study highlighted differential expression of genes involved in muscle contraction (skeletal and cardiac) (Arcanjo et al., 2018), 24 hpf embryos to 96 hpf larvae were challenged with behavioral analyses including spontaneous tail movements, heart rate and free-swimming behavior. Histological observations of eyes from 48 hpf to 96 hpf were also performed to make the link with modulation of genes involved in eye transparency. Finally, a whole mount immunostaining was performed to detect gamma-H2AX foci, an early marker of double strand break repair process (Rogakou et al., 1998), as some genes involved in DNA repair pathways were found mis-regulated in a previous study (Arcanjo et al., 2018).

2. Materials and methods

2.1. Fish maintenance and eggs production

Adult wild type zebrafish (AB genetic background) were maintained in a Tecniplast© ZebTEC system in 8L tanks (20 adults per tank) with a temperature of 28°C, males and females being kept separated. A photoperiod of 12/12h dark-light cycles was used. Eggs were produced by mating couples in 1.7L Breeding Tank Beach Style Design (Tecniplast©) in a thermoregulated room at 28°C. Males and females were kept apart in the same breeding tank during the night before the mating. The morning of mating, couples were allowed to mate, and eggs were sampled 30 min after the beginning of the mating in order to obtain synchronised eggs. All eggs were pooled together, sorted, counted and kept in petri dish with embryo medium (60mg/L of Instant Ocean© sea salt diluted in ultrapure water (Westerfield, 1995)), at 28 ±0.5°C until the beginning of the experiments.

2.2. Eggs contamination and sampling

Tritiated water source (97% purity, 185 MBq, PerkinElmer, Courtaboeuf, France) was diluted in embryo medium to obtain final activity concentrations of 1.22×10^5 and 1.22×10^6 Bq/mL. At 3 hpf, eggs were contaminated respecting a density of 5 eggs per 2 mL of HTO solution in three experimental units (three replicates). To perform the behavioural and physiological experiments, the histological sections and the whole mount immunostaining, 1620 eggs were used for both conditions, control and HTO-contaminated (135 eggs * 3 replicates * 2 treatments * 2 dose rates). The eggs were kept in the dark at 27.7 ± 0.3°C in a thermo-regulated incubator confined inside a glove box. Control eggs were placed in embryo medium and kept in the same manner as contaminated organisms (27.8 ± 0.07°C) in the same room outside the glove box. Water in contaminated and control experimental units was renewed once (at 72h) during the experiment to ensure water quality and constant tritiated water activity. Samples of contamination medium were made to monitor tritium activity along the experiments in the three experimental units. These samples were analysed by liquid scintillation counting. Briefly, samples were diluted with tritium-free water (Les Abatilles, Arcachon, France) to obtain a volume of 1 mL. A liquid scintillation cocktail (Ultima Gold LLT, PerkinElmer) was then added to a final volume of 20 mL. Samples were analysed using a Quantulus 1220 (DL = 5 Bq/L, PerkinElmer, WinQ software) and spectrum were analysed using the EASY View Spectrum analysis Program (PerkinElmer). To avoid cross-contamination between the two HTO activity concentrations, experiments were conducted

separately. The statistical analyses performed to compare mean activity concentration between experimental units showed no differences for both activities tested in this study. Measured activity concentrations along experiments and for all experimental units were of $1.10x10^5$ ($\pm 5.72x10^3$) Bq/mL and $1.35x10^6$ ($\pm 8.89x10^4$) Bq/mL respectively for the lowest and the highest activity concentration tested.

Concerning the sampling of organisms needed for the behavioral and histological analyses as well as for the gamma-H2AX immunostaining, contaminated organisms were washed three times in non-tritiated embryo medium to eliminate tritium contamination around the eggs that could lead to over-estimate radiological dose. Control organisms were handled in the same way. This protocol was optimised for zebrafish (Arcanjo et al., 2019).

2.3. Behavioral analyses

All behavioral analyses were preceded by an acclimation period of 30 min for all organisms (control and contaminated) in the behavioral room. Acclimation was performed in a dark thermoregulated incubator (AquaLytic, Germany) at 28 ± 1°C. Videos for the assessment of spontaneous tail movements of chorionated embryos at 24 hpf and heart rate of 48 hpf and 72 hpf larvae were performed under a stereomicroscope (Nikon SMZ800) connected to a high-resolution camera (acA1300-60gm, Basler, Germany) under the behavioural room light and temperature (28°C). Recording were performed using Media Recorder Software (v.4.0.542.1, Noldus Information Technology, Netherlands) and the analysis of videos was performed using the DanioScope Software (v.1.1.110, Noldus Information Technology, Netherlands). Concerning the swimming behavior of 96 hpf larvae, the DanioVision Observation Chamber (Noldus Information Technology, Netherlands) coupled with the DanioVision Temperature Control Unit (Noldus Information Technology, Netherlands) at 28°C (± 0.1) were used and videos were analysed using the EthoVision XT software (v.13.0.1216, Noldus Information Technology, Netherlands). All videos used for the swimming behavior analyses were performed in the dark under infra-red light. Results for all behavioral tests were compared to their respective control.

180 *2.4.1. Spontaneous tail movements of 24 hpf embryos*

181 For the assessment of the spontaneous tail movement in 24 hpf embryos, three
182 replicates of 30 organisms were used. As the 30 organisms cannot be recorded together
183 under the stereomicroscope, replicates were divided in three sub-replicates of 10 organisms.
184 After the acclimation period, sub-replicates were placed on a petri-dish containing 1%
185 agarose gel (Sigma-Aldrich), then 20 µL of methyl cellulose was added to avoid movement of
186 eggs during the recording time. Organisms were then placed under the stereomicroscope
187 and after an acclimation period of 2 min before the videos were recorded (5 min of video,
188 frame rate = 25 Hz). Control and contaminated replicates were alternated. All the recordings
189 of the spontaneous movement in 24 hpf embryos took place between 10:20 am and 01:00
190 pm. For the statistical analysis, the burst activity was used. It corresponds to the percentage
191 of time the embryo was moving along the duration time of the video. Based on the data
192 distribution, values below 1 and above 40 were considered as outliers and were excluded
193 from the data set.

194

195 *2.4.2. Heart rate of 48 hpf and 72 hpf larvae*

196 For the heart rate analysis, protocol was almost the same as described for the activity
197 assessment, except that three replicates of 10 organisms were used for each stage. These
198 replicates were divided in three sub-replicates of 5 organisms. After the acclimation period,
199 sub-replicates were placed on a petri-dish containing 1% agarose gel (Sigma-Aldrich), then
200 20 µL of methyl cellulose and 1 µL of MS-222 final concentration 0.02 % (w.v.) (Tricaine
201 methanesulfonate, Molekula) were added to avoid movement of larvae during the recording
202 time. After the end of video, larvae were placed in clean embryo medium and started to
203 swim normally. When anesthetised, organisms were then placed under the
204 stereomicroscope and, after an acclimation period of 2 min, the videos were recorded (30
205 sec of video, frame rate = 25 Hz). Control and contaminated replicates were alternated. All
206 the recordings of the heart rate in 48 hpf and 72 hpf larvae took place between 10:15 am
207 and 11:30 am. For the statistical analysis, based on the data distribution, a number of beats
208 per second below 1.5 and above 3.5 were considered as outliers and were excluded from the
209 data set.

2.4.3. Swimming behavior of 96 hpf larvae

The swimming behavior of the 96 hpf larvae was assessed using 48 organisms per treatment distributed in four 24-wells plates. Organisms were placed individually in each well with 2 mL of embryo medium; controls and contaminated animals were placed in the same plate. All four plates were prepared few minutes before the beginning of their respective recording time. Once placed in the DanioVision Observation Chamber, an acclimation period of 10 min was respected before the beginning of the recording. Videos of 10 min were recorded with a tapping stimulus (level=8, on the side of the well plate) at t+5 min after the beginning of the recording. All the recordings of the swimming behavior in 96 hpf larvae took place between 11:00 am and 12:40 pm. Analysis were made between control and contaminated during the first five minutes of the video to assess the swimming activity basal level. The response to the tactile stimulus was assessed by comparing responses 1 second before and 1 second after the stimulus for all treatment. Then the delta of the response (after-before) was used to compare the response to the tactile stimulus between control and contaminated larvae.

2.5. Histological analysis of eyes

The histological sections of zebrafish eyes from 48 hpf to 96 hpf were performed after the exposure to 4 mGy/h of HTO only. Sections of 400 nm were realized using an ultramicrotome UCT (Leica Microsystems GmbH, Wetzlar, Germany) and stained with toluidine blue. Sections were then observed with an optic microscope (DM750, Leica) with ICC50 camera (Leica) coupled with the LAS EZ software. Images focusing on retina and lens were made using the 100x objective. Both eyes for two larvae were used for the three stages studied representing 12 organisms.

2.6. Whole mount gamma-H2AX immunostaining

2.6.1. Immunostaining

The whole mount gamma-H2AX immunostaining were performed using at least 20 embryos at 24 hpf for each treatment (control and contaminated). After the sampling, eggs were manually dechorionated and fixed in 2 mL of PFA 4% in PBS 1X (Sigma-Aldrich) during 3 h at 4°C. Once fixed, embryos were kept in 100% methanol (VWR International) at -20°C until the beginning of the staining.

The staining begins by the rehydration of embryos with successive washes with decreasing concentrations of methanol in PBS 1X (70%; 50%; 25% methanol). Embryos were then permeabilized with Proteinase K in PTW buffer (10 µg/mL final concentration) for 3 min at room temperature. After a brief wash in PTW buffer, they were fixed in PFA 4% in PBS 1X (Sigma-Aldrich) for 20 min at 4°C. The samples were blocked in a 0.2% BSA and 1% DMSO PBST solution for 3 h at room temperature, and then incubated with the primary antibody (1:200 gamma-H2AX in blocking solution, GeneTex GTX127342) at 4°C overnight. The samples were washed five times in PTW buffer, incubated with the secondary antibody (1:500 Sigma-Aldrich anti rabbit-FITC F0382) 2 h at room temperature and then accurately washed with PTW buffer. The samples were kept protected from light at 4°C until analysis by confocal microscopy.

2.6.2. Confocal observations

Confocal microscopy analyses were performed using a laser scanning confocal microscope (Carl Zeiss, France) using a 20x dry objective (N.A. 0.8), with a pinhole setting of 1 A.U. For FITC fluorescence, excitation was performed at 488 nm and emission acquired between 510/550 nm. For DAPI detection, samples were stained in a 0.1% Tween and 300nM DAPI PBS solution for 1 h and 30 min, excitation was performed at 405 nm and detection between 420 and 500 nm. The samples were mounted on 35 mm imaging dish with a polymer coverslip bottom (Ibidi) and covered by a 2% methyl-cellulose solution.

2.6.3. Image analysis

The images acquired at the confocal microscope were analysed using the open-source software Fiji ImageJ. For each image, the smooth filter was applied (this filter replaces each pixel with the average of its 3×3 neighbourhood) to increase the ratio

signal/noise, and then the level of background was chosen (values between 5 and 10). Several regions of interest (ROI) were designed on the nuclei detected in the DAPI channel, using the ImageJ tool ROI manager. The ROI were transferred and combined with the FITC channel. The function Find Maxima was used to identify and count the foci; this algorithm determines local maxima that pass the threshold (noise tolerance) in the selected area. The values derived from the initial background analysis were used as a threshold. The ratio between the number of maxima identified by the function and the number of selected nuclei was reported as foci per nucleus.

2.7. Statistical analyses

All statistical analyses were performed using the R software (v 3.4.3) (R Core Team, 2017). Behavioral parameters, i.e. tail spontaneous movements, heart rate and locomotor activity, were analysed using linear mixed effect models fitted with the *nlme* package (Pinheiro et al., 2017). Treatment and time (at which the video was recorded reported as "time effects" in the text) were included as fixed effects while replicates were included as random effects. Residuals normality and homogeneity assumptions were assessed employing QQplot and fitted values vs. standardized residuals plot. Since they were not satisfied random permutation tests were finally applied using the *pgirmess* package (Giraudoux et al., 2018).

HTO activity in water and gamma-H2AX foci were analysed using Student t tests, or Wilcoxon tests if validity condition were not satisfied, and using two ways ANOVA. Normality and homogeneity assumptions were assessed using Shapiro-Wilk tests and Bartlett tests respectively. Hypotheses were bilateral, and significance level was set to 0.05.

3. Results

3.1. Behavioral and physiological analysis

In 24 hpf embryo, the spontaneous movements of the tail were not significantly different in controls and in contaminated organisms for both dose rates (Figure 1). A

296 significant effect of the time was found for both dose rates and treatment as a diminution of
297 tail movement (data not shown).

298 The heart rate of 48 hpf and 72 hpf larvae were also analysed. Beats per second (BPS)
299 in control and contaminated organisms were not significantly different for both stages and
300 dose rates (Figure 2). A significant effect of the time was highlighted for both dose rates in
301 48 hpf larvae and only for 4 mGy/h in 72 hpf larvae as an increase of BPS (data not shown).

302 Concerning the swimming behavior of 96 hpf larvae after a 0.4 mGy/h exposure,
303 contaminated organisms showed a significant lower velocity (mm/s) during the first five
304 minutes of the tracking videos than control organisms (Figure 3A). This difference was not
305 found in 96 hpf larvae exposed to 4 mGy/h (Figure 3B). A second experiment was conducted
306 with 96 hpf larvae exposed at 0.4 mGy/h and the same trend was highlighted (Figure S1).
307 Following the tapping stimulus (i.e. one second before and one second after), control and
308 contaminated 96 hpf larvae showed significant differences in velocity (mm/s). Indeed, the
309 velocity of 96 hpf larvae was significantly lower one second before the tapping stimulus than
310 one second after in control and contaminated organisms for both dose rates (Figure 4). The
311 delta of the response to the stimulus (i.e. after-before) between control and contaminated
312 organisms was compared for both dose rates. Results showed that after an exposure to 0.4
313 mGy/h, the delta of the response in contaminated 96 hpf larvae was significantly lower than
314 the delta observed in control organisms (Figure 5). After an exposure to 4 mGy/h, no such
315 difference was found (Figure 5).

316

317 ***3.2. Histological observations of eyes***

318 The histological observations were performed on larvae eyes from 48 hpf to 96 hpf
319 exposed to a dose rate of 4 mGy/h. The comparison between pictures did not show notable
320 alterations in the retinal structure when comparing control and contaminated organisms for
321 all stages (Figure S2). Indeed, as expected when normal development occurs, the fives layers
322 composing the retina were not distinguishable from each other in 48 hpf larvae but well
323 defined in 72 hpf and 96 hpf larvae (Figure S2). When focusing on the lens, no remarkable
324 difference appeared in lens development between 4 mGy/h exposed larvae and controls for
325 all the three stages. In 48 hpf larvae, lens was fully detached from the ectoderm and showed

a mono layer of epithelial cells on the anterior part of the lens while in the central part elongated primary lens fibre were visible as a concentric layer (Figure 6). On later stages (i.e. 72 hpf and 96 hpf larvae), the lens structure became more homogenous, with cells forming a concentric structure still visible in 72 hpf larvae but not in 96 hpf larvae (Figure 6).

3.3. Whole mount gamma-H2AX immunostaining

For both dose rates, the comparison between control and contaminated organisms was performed using the foci per nucleus ratio. For control and samples exposed to 0.4 mGy/h, a total of 850 and 779 cells were counted, respectively. A similar number of cells were counted in control and 24 hpf embryos exposed to 4 mGy/h (824 for control and 936 for contaminated samples). For both dose rates, no significant difference was highlighted between control and contaminated 24 hpf embryos (Figure 7).

4. Discussion

4.1. Behavior and heart rate

4.1.1. HTO exposure did not change spontaneous tail movement

At 24 hpf, the spontaneous movement of the zebrafish embryo tail was investigated. Results did not show significant differences between control and contaminated organisms for both dose rates. These results suggested that genes coding for proteins involved in muscle contraction found, via a transcriptomic analysis, to be affected in 24 hpf eggs for both dose rates (Arcanjo et al., 2018) did not induce any effect on spontaneous tail movement at the same developmental stage. The spontaneous movements of the tail originate from the spinal cord and are dependant of functional motoneuron innervation (Brustein et al., 2003) as well as muscle contraction. Furthermore, a well-known neurotoxicant such as chlorpyrifos was shown to increase tail movements in zebrafish embryo from 24 to 26 hpf from 0.625 to 10 mg/L (Selderslaghs et al., 2010). Our result suggests that this pathway did not seem to be affected by HTO exposure in 24 hpf eggs for both dose rates tested, questioning the neurotoxic effect of HTO. However, genes coding for proteins involved in neurotransmitter and Ca^{2+} transport were modulated in 24 hpf eggs

after exposure to 0.4 and 4 mGy/h of HTO (Arcanjo et al., 2018). Furthermore, in 24 hpf eggs exposed to 4 mGy/h of HTO an over-expression of the *ache* gene, coding for the acetylcholinesterase protein that play a role in muscle relaxation or muscular and neuronal development (Behra et al., 2002), was found compared to control (Gagnaire et al, in prep). Together, these results suggest that the neuro-muscular functions could be impaired after HTO exposure and it would be interesting to perform further investigations with older developmental stages. Indeed, as genes mis-regulation were found at 24 hpf, it was probably too soon to see any phenotypical impairments at the same time. That is why we also performed locomotor analysis on 96 hpf larvae to assess the latency between the gene expression impairments and the phenotypical observations.

Interestingly, the statistical analysis performed on the spontaneous tail movement highlighted a significant effect of time for both HTO exposures; meaning that the time during which experiments were made has a significant effect on the measured parameter (burst activity). Indeed, the first spontaneous movement appeared around 17 hpf. In dechorionated embryos, the spontaneous movements reach a peak around 19 hpf and then slowly decrease by 26 hpf (Saint-Amant, 2006). Such a decrease of the spontaneous tail movement was also reported in literature in control organisms (Huang et al., 2010; Wang et al., 2013) confirming that time influence this parameter. In the present study, as experiments were performed on stages between 24 hpf and 27 hpf, it is not surprising to find a significant effect of time in the obtained data.

4.1.2. HTO exposure did not change the number of beats per seconds

After an exposure to 0.4 mGy/h of HTO, no significant differences in BPS were highlighted between control and exposed organisms for both stages. The same result was found for the highest dose rate of 4 mGy/h. Furthermore, the statistical analyses showed a significant effect of time for both dose rate when 48 hpf larvae were investigated and only for the highest dose rate for the 72 hpf larvae suggesting that the time at which the experiments are recorded could influence the heart rate. Together, the results found for the cardiac activity after HTO exposure suggested no effect of HTO on this parameter for the developmental stages investigated. As previously said, it appears that the down-regulation

of genes involved in both muscle contraction and event leading to the muscle contraction found in a previous study in 24 hpf eggs (Arcanjo et al., 2018) had no effects on cardiac activity on 48 hpf and 72 hpf larvae. These results could indicate that the organisms can cope with tritium toxicity with early gene expression changes, indicating the low toxicity of tritium, at least for the considered life stages and exposure period. Using gamma radiation, it is possible to observe effects on heart rate with dose as low as 0.5 mGy/h in 48 hpf larvae (Murat El Houdigui et al, under review). To go further, it would be interesting to perform dedicated experiments to find the moments of the day were larvae show constant BPS to avoid time effect.

4.1.3. HTO exposure induces effects on locomotor behavior

To get insight into the swimming abilities of 96 hpf larvae after HTO exposure, a free-swimming behavioral test was performed in 96 hpf larvae, followed by a tactile stimulus on the side of the plate containing organisms. Results highlighted that during the first five minutes of the video tracking, the 0.4 mGy/h exposed larvae showed a mean velocity significantly lower than the velocity of control larvae. The response to a tactile stimulus was then investigated and showed that controls and HTO-contaminated organisms (for both dose rates) responded to the tactile stimulus by a significant enhancement of their velocity one second after the stimulus. The delta of the response to the tactile stimulus was also studied. It appeared that the 0.4 mGy/h exposed 96 hpf larvae showed a delta of the response significantly lower than control organisms.

The effects found on 96 hpf larvae locomotor activity could be explained by the potential impact of HTO exposure on muscle structure and events leading to muscle contraction. Indeed, muscles impairments as disorganization of sarcomeres myofilaments (96 hpf) and disruption of genes involved in muscle contraction (24 hpf for both dose rate) were reported in a previous study using the same HTO dose rates (Arcanjo et al., 2018). Also in zebrafish, gamma exposure to 0.03 mGy/h and 24 mGy/h induced degradation of myofibrils which were more deleterious for the higher dose rate tested (Gagnaire et al., 2015). In 96 hpf larvae TEM muscle observation reveals myofibers and neuromuscular junction alterations after gamma irradiation at 5 and 50 mGy/h (Murat El Houdigui et al,

under review). Furthermore, after HTO or gamma exposure, vertebral malformations have been documented in medaka, *Oryzia latipes*, exposed to 35 mGy/h (HTO) and 18 mGy/h (γ) during embryonic stages (Hyodo-Taguchi and Etoh, 1993) which could lead to locomotor impairments. To explain the locomotor effects of HTO on zebrafish larvae, it would be also interesting to focus on the lateral line, a mechanosensory system in fish and on the Mauthner cells which mediated the trunk and tail contraction during the response to a tactile stimulus (Eaton et al., 1977). Indeed, after uranium exposure, severe tissue alterations where reported in the lateral line system in zebrafish (Faucher et al., 2012).

However, the impairments of the muscular system, both at the molecular and tissue scales, cannot explain the observed effects alone, as the locomotor activity in 96 hpf larvae exposed to 4 mGy/h was not affected by HTO exposure. As a proper locomotor activity depends on both the muscular and the nervous systems (Drapeau et al., 2002), the potential neurotoxic effect of HTO exposure has also to be considered. After gamma irradiation, zebrafish larvae show neuronal as well as muscles impairments (Murat El Houdigui et al, under review). After HTO exposure, a transcriptomic analysis highlight down-regulation of genes coding for proteins involved in peripheral nervous system axonogenesis (*her4.3* and *her4.4*) in 24 hpf embryos after exposure at 4 mGy/h (Arcanjo et al., 2018). In studies focusing on chemicals such as nicotine (Parker and Connaughton, 2007; Svoboda et al., 2002), caffeine (Chen et al., 2008) or ethanol (Carvan et al., 2004), effects on the locomotor behavior of zebrafish larvae after a tactile stimulus, dose dependant decrease of response was shown in contaminated organisms comparing to controls. The mode of action evoked to explain these results are linked to an effect on the development and on the alteration of the axonal pathfinding of the secondary spinal motoneurons as defects on the neuromuscular junction (Chen et al., 2008; Svoboda et al., 2002).

Together, these results suggest that HTO exposure had more detrimental effect after an exposure to 0.4 mGy/h than to 4 mGy/h on the locomotor activity in 96 hpf larvae. This lack of concentration-dependant response could be due to the onset of more efficient defence mechanisms following a threshold that is not reach for the lowest dose rate. Indeed, in a previous study, we found more differentially expressed genes involved in DNA repair pathways at 4 mGy/h than at 0.4 mGy/h (Arcanjo et al., 2018). However, at 0.4 mGy/h, the events triggering the impairment of the locomotor activity in larvae remained to be

confirmed. Indeed, the velocity of the control larvae in the 0.4 mGy/h experiment was higher than the velocity in control larvae for the experimentation with the highest dose rate. A second experiment were performed at 0.4 mGy/h, and the same trend was highlight with a mean velocity in control of the same range as for the first experiment. The difference in control larvae mean velocity between dose rates could be explained by biological variation between the pool of eggs used. Indeed, to avoid HTO cross-contamination, the experiments for both dose rate were conducted separately in time, meaning that pools for both experiments were not from the same spawn. This could lead to find a significant difference in velocity where there is not. Nevertheless, the impact of HTO on the velocity of larvae could cause delayed response to threats by impairing the escaping/avoidance behavior or decrease success of prey capture and could therefore have a detrimental impact on fish fitness.

In the literature, it is known that many variables could influence zebrafish larvae locomotor behavior, i.e. larvae age, size of well, time of the day or experiment performed in light or dark (MacPhail et al., 2009; Padilla et al., 2011). In the present study, we chose to assess locomotor activity on 96 hpf larvae to be consistent with experiments on gene expressions where genes involved in muscle contraction were mis-regulated in 24 hpf embryos. Furthermore, in locomotor activity experiments performed in the dark, as in the present study, response in 4 dpf, 5 dpf and 6 dpf larvae are similar to each other, which is not the case in light experiments (Padilla et al., 2011), reinforcing the use of 96 hpf larvae in our study. Time of the day when the experiments are performed could also influence larvae locomotor response. Indeed, in 6 dpf larvae, the locomotor activity assessed in the dark reached stability in early afternoon around 01:00 pm (MacPhail et al., 2009). Even though our experiments were performed in late morning (from 11:00 am to 12:40 pm) when it seems that locomotor activity is still stabilizing, we were able to find effects of HTO exposure on larvae velocity.

4.2. Histology of the eyes

As cataract is one of the complications after beta radiation therapy in Human and that cataract incidence was shown to increase in Chernobyl birds (Barron et al., 1970;

Mousseau and Møller, 2013), the potential development of cataract in HTO exposed zebrafish larvae was investigated. Furthermore, as irregularities in the development of zebrafish larvae eye were reported after gamma radiation in zebrafish larvae exposed only via their parents, exposed only as larvae or both (Hurem et al., 2017), eye layer development was assessed. Histological sections of eyes lens and retina were performed. As genes involved in eye transparency were found down-regulated in 24 hpf eggs after an exposure to 4 mGy/h in a previous study (Arcanjo et al., 2018), all sections were made at this dose rate. Results highlighted no distinguishable defects in larvae (from 48 hpf to 96 hpf) when comparing control and contaminated organisms. Indeed, as expected in normal zebrafish eyes development, in 48 hpf larvae the lens is fully detached from the cornea and show different kind of cells, an anterior monolayer of epithelial cells and in more centrally the primary lens fibre starting to elongated (Dahm et al., 2007). At 72 hpf, histological sections of lens showed a more homogeneous appearance with lens fibre cell even more elongated with degraded nucleus (Dahm et al., 2007). Finally, at 96 hpf, sections of the lens also showed a homogenous pattern with no appearance of nuclei. At this stage the differentiation of lens fibre cells is terminated, and lens is totally transparent (Goishi et al., 2006). Concerning the histological sections of retina, no obvious differences could be seen between control and contaminated organisms regardless of stages. Indeed, the five retinal layers were well distinguishable in 72 hpf and 96 hpf larvae. These five layers are composed of three nuclear layers (ONL, INL and GCL) and two plexiform layers (IPL and OPL) (Chhetri et al., 2014). No remaining nuclei was found in lens, as already shown by histological section of zebrafish eyes with cataract (Dhakal et al., 2015; Goishi et al., 2006). Therefore, these results did not support the molecular alterations previously shown (Arcanjo et al., 2018).

Together, the results showed no effect of HTO on lens transparency or retinal development at the analysed developmental stages. This results suggest that the transient down-regulation of genes involved in eye transparency at 24 hpf (Arcanjo et al., 2018) did not have any effect on eye development. Another hypothesis would be that toxico-dynamics are different between molecular and tissular effects. To check this last hypothesis, it would be interesting to follow eye development over the whole larvae development until the adult stage. In addition, considering the observed behavioural changes, it would be an asset to investigate behavioral tests dedicated to visual parameters as the optokinetic response

(OKR) or the optomotor response (OMR) in 96 hpf larvae. Indeed, at this stage the retinal structure and the ocular muscles are functional, which leads to visible eye movements which could indicate a behavioral changes, liable to occur even in the absence of any structural defects (Chhetri et al., 2014). Indeed, the OKR was used to isolated zebrafish mutant with visual defect and highlighted two mutants lacking obvious morphological difference from controls (Brockerhoff et al., 1995).

4.3. gamma-H2AX foci detection

The effects of HTO exposure on DNA damages were already investigated using a wide range of species as mollusc (Hagger et al., 2005; Jha et al., 2005), aquatic vertebrates (Gagnaire et al., 2017; Suyama et al., 1981) or fish cell culture (Stuart et al., 2016). In the present study, we focused on DNA double strand breaks in 24 hpf embryos using an immunostaining method to assess the number of gamma-H2AX foci, an early marker of DNA double strand break repair (Rogakou et al., 1998). Results showed that, comparing to their respective controls, no significant differences were found for both dose rates. After an exposure to tritium at much lower activities than in the present study, no difference between control and contaminated organisms were neither found in fish cells and *in situ* exposed fathead minnows (Gagnaire et al., 2017; Stuart et al., 2016) using gamma-H2AX foci detection. At the same stage, i.e. 24 hpf, no DNA damages were found using the comet assay for both dose rates (Gagnaire et al, in prep). Using fish cells (3B11 and FHMT-W1), no effects of HTO exposure was found when investigating the gamma-H2AX foci intensity after exposure from 0.01 to 100 Bq/mL (Stuart et al., 2016). After gamma irradiation, no significant difference in the gamma-H2AX detection was found after exposure at dose rate close to those in the present study (5 mGy/h) for the same stage while an increase of foci was detected at 50 mGy/h (Murat El Houdigui et al, under review). By exposing directly ZF4 zebrafish cells to chronic gamma radiation, an increase of gamma-H2AX foci per nucleus was found after 24h at 4 mGy/h while no effects was found at 0.4 mGy/h (Pereira et al., 2011).

Overall, the results found in the present study combined with those in studies investigating gamma-H2AX foci detection after exposure to HTO or gamma radiation suggest that organisms could cope with DNA damages potentially occurring after an exposure to

ionizing radiations. These results could be due to an enhancing of DNA repair pathways at those dose rates to balance the effects of ionizing radiations. This hypothesis is consistent with previous findings at the molecular scale highlighting an up-regulation of genes involved in DNA repair pathways, in 24 hpf eggs after an exposure to HTO at 4 mGy/h (Arcanjo et al., 2018). However, this hypothesis remains to be confirmed by further investigation on the DNA repair pathways after ionizing radiation exposure.

5. Conclusion

In this study, observed effects at the molecular scale, i.e. genes involved in muscle contraction, eye transparency and response to DNA damage, have not all been confirmed at higher biological scales using stages from 24 hpf to 96 hpf. However it was possible to confirm these early effects for locomotion. Results highlighted that the 0.4 mGy/h exposure could have more detrimental effects that the 4 mGy/h exposure on the locomotor activity. These results could be partly explained by the impact on muscle (structure and genes) previously highlighted for both dose rate, but not entirely. Therefore, an impact on the nervous system coupled with muscular impairments could be more relevant for the explanation of effects on the locomotor behavior. Furthermore, the ability to cope with damages after HTO exposure could also be advanced to explain the observed effect on locomotion. Concerning the other parameters followed in this study, it could be interesting to explore the responses by using older larvae as there may be a delay between gene expression changes and the onset of physiological disruption or homeostatic mechanisms onset at those early stages which could be exceeded along time. Despite that, the present study and the whole project related to, allowed to link responses observed at different organization levels, from molecule to individual, contributed to improve the knowledge of tritiated water exposure effects. From an ecological risk assessment point of view, the present study did not highlight detrimental effects at the individual scale with tritium activity as high as 1.22×10^9 Bq/L. Activities used in the present study are widely higher than those found in aquatic environment suggesting that environmental exposure to tritium could lead to poor detrimental effects in organisms. However, it is not excluded that chronic exposure to environmental activities of tritium could lead, over time, to detrimental

effects at the individual level. Indeed, at the same activities as is the present work, molecular effects were highlight and could, with time, generate detrimental effects.

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Figure 1: Burst activity in control (white bars) and HTO-contaminated (grey bars) 24 hpf embryos. The left bar plot represents control and contaminated 24 hpf embryos exposed at 0.4 mGy/h. Right bar plot represents control and HTO-contaminated 24 hpf embryos exposed at 4 mGy/h (Permutation test pvalue: * < 0.05)

Figure 2: Heart-beats per second in control (white bars) and HTO-contaminated (grey bars) organisms. 48 hpf larvae were exposed at 0.4 mGy/h (top left) and 4 mGy/h (bottom left). 72 hpf larvae were exposed at 0.4 mGy/h (top right) and 4 mGy/h (bottom right) (Permutation test pvalue: * < 0.05)

Figure 3: Bar-plot of the comparison between the larvae basal velocity (mm/s) of control (white bars) and HTO-contaminated (grey bars) 96 hpf larvae after exposure at (A) 0.4 mGy/h and (B) 4 mGy/h of HTO (Permuation test pvalue: * < 0.05)

Figure 4: Comparison between the velocity (mm/s) of 96 hpf larvae one second before (white bars) and one second after (grey bars) the tactile stimulus. The left bar-plot represents control and contaminated 96 hpf larvae exposed at 0.4 mGy/h. The right bar-plot represents control and contaminated 96 hpf larvae exposed at 4 mGy/h (Permutation test pvalue: * < 0.05)

Figure 5: Bar-plot representing the delta of the response to the tactile stimulus in control (white bars) and contaminated (grey bars) in 96 hpf larvae. The left bar-plot represents control and contaminated 96 hpf larvae exposed at 0.4 mGy/h. The right bar-plot represents control and contaminated 96 hpf larvae exposed at 4 mGy/h (Permutation test pvalue: * < 0.05). Delta = after-before

Figure 6: Histological sections of control (left) and contaminated (right, 4 mGy/h) lens of 48 hpf, 72 hpf and 96 hpf larvae (from top to bottom). At 48 hpf (top), the lens is formed of a monolayer of epithelial cells (dashed yellow lines) and primary lens fibers (PLF) with visible nucleus. The cornea (C), the vitreous cavity (V) and blood vessels (BV) are also visible. At 72 hpfn the lens fiber shows a more elongated pattern and at 96 hpf, the lens (L) show a more homogeneous pattern. In all panels, ventral is down

Figure 7: Detection of gamma-H2AX foci per nucleus in 24 hpf embryos by whole mount-immunostaining. Comparison of control organisms (white bars) and HTO-contaminated organisms (grey bars). Embryos exposed at 0.4 mGy/h are represented in the left bar plot and those exposed at 4 mGy/h in the right bar plot (Wilcoxon pvalue: * < 0.05)