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Characterization of the adenosinergic system in a zebrafish embryo radiotherapy model

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ABSTRACT

Adenosine is a nucleoside that acts as a signaling molecule by activating P1 purinergic receptors (A_1, A_{2A}, A_{2B} and A_3). This activation is involved in immune responses, inflammation, and tissue remodeling and tumor progression. Gamma rays are a type of ionizing radiation widely adopted in radiotherapy of tumors. Although it brings benefits to the success of the therapeutic scheme, it can trigger cellular damages, inducing a perpetual inflammatory response that culminates in adverse effects and severe toxicity. Our study aims to characterize the adenosinergic system in a zebrafish embryo radiotherapy model, relating the adenosine signaling to the changes elicited by radiation exposure. To standardize the radiotherapy procedure, we established a toxicological profile after exposure. Zebrafish were irradiated with different doses of gamma rays (2, 5, 10, 15 and 20 Gy) at 24 hpf. Survival, hatching rate, heartbeats, locomotor activity and morphological changes were determined during embryos development. Although without significant difference in survival, gamma-irradiated embryos had their heartbeats increased and presented decreased hatching time, changes in locomotor activity and important morphological alterations. The exposure to 10 Gy disrupted the ecto-5′-nucleotidase/CD73 and adenosine deaminase/ADA enzymatic activity, impairing adenosine metabolism. We also demonstrated that radiation decreased A_{2B} receptor gene expression, suggesting the involvement of extracellular adenosine in the changes prompted by radiotherapy. Our results indicate that the components of the adenosinergic system may be potential targets to improve radiotherapy and manage the tissue damage and toxicity of ionizing radiation.

1. Introduction

The purinergic system represents the machinery that allows nucleotides and nucleosides to exert their cellular signaling (Burnstock, 2017). It is comprised of two receptor families: P1 receptors (P1R) and P2 receptors (P2R), categorized as P2XR and P2YR subtypes (Jacobson and Müller, 2016). Adenosine binds and activates the four P1R subtypes (A_1, A_{2A}, A_{2B} and A_3), which are G-protein coupled receptors broadly expressed throughout the body and involved in several physiological functions (Chen et al., 2013; Fredholm, 2010). Also crucial components of this system, the enzymes that metabolize ATP and ADP to AMP (CD39 or E-NTPDase); AMP to adenosine (CD73, ecto-5′-nucleotidase or ecto-5′-NT) and adenosine to inosine (adenosine deaminase or ADA), are responsible for regulating the extracellular levels of these signaling molecules (Di Virgilio, 2012).

It has been widely described the fundamental role of adenosine in the contexts of inflammation and cancer (Antonioli et al., 2013). In response to hypoxia and tissue damage, the accumulated adenosine in the inflammation site can regulate the inflammatory responses, promoting tissue remodeling, angiogenesis and resolution of inflammation (Ohta, 2016). By its immunosuppressive action, adenosine in high levels in the tumor microenvironment may be beneficial for tumor progression, inhibiting antitumor immunity, and being involved in the processes of differentiation, proliferation and apoptosis (Antonioli

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et al., 2013; Gessi et al., 2011). Vaupel and Multhoff (2016) have reported that adenosine may prevent antitumor immune responses elicited by radiotherapy and suggests mechanisms to avoid these effects. However, little is known about the involvement of adenosine in the events related to toxicity and response to radiation treatment.

Radiation therapy is known to cause cell death through DNA breakage, generation of reactive oxygen species (ROS), release of cytokines, chemokines and growth factors, and can also trigger an inflammatory response with consequent tissue damage (Hekim et al., 2015; Schaue et al., 2015). Another important radiobiological effect is the immunogenic cell death, a type of tumor cell death that involves the activation and recruitment of the host's immune system cells, engaging an antitumor immunity and contributing to radiosensitization (Golden and Apetoh, 2015; Rodriguez-Ruiz et al., 2019). Even though it helps crucially in the eradication of tumors by several mechanisms of action, radiotherapy can also cause an important toxicity scenario due to exacerbated and perpetual inflammation (Sprung et al., 2015).

Zebrafish (*Danio rerio*) presents several characteristics similar to mammals (Goldsmith and Jobin, 2012) and expresses the receptors and enzymes that compose the purinergic system since its early life stages (Boehmler et al., 2009; Cruz et al., 2017; Leite et al., 2013). It has been adopted in several phases of development, as a model for the study of gamma radiation toxicity (Hu et al., 2016; Hurem et al., 2017; Pereira et al., 2011; Praveen Kumar et al., 2017), radiotherapy response (Geiger et al., 2008; Lally et al., 2007) and radioprotection (Dimri et al., 2015a, 2015b; Geiger et al., 2006). Considering that: (i) radiotherapy is crucial in tumors treatment with a direct effect on the immune system and can trigger chronic inflammation; (ii) adenosine is involved in immunity, inflammation and cancer; and (iii) zebrafish is a suitable model for the study of the adenosine signaling, our study aims to characterize the adenosinergic system in a zebrafish embryo radiotherapy model. We also related the adenosine signaling to the toxicological changes elicited by radiation exposure.

2. Materials and methods

2.1. Zebrafish maintenance

Adult zebrafish were maintained in an integrated aquarium system (Zebtec, Tecniplast®, Italy) with controlled water conductivity, temperature and pH, under a light/dark cycle of 14/10 h. Animals diet was based on feeding with commercial flake and artemia (*Artemia salina*). To obtain the embryos used in the following experiments, fishes were mated as described by Westerfield (2000). Embryos were selected and allocated in 12 well plates, with 5 embryos per well in a final volume of 3 mL of medium. The plates were maintained in greenhouse B.O.D (Biochemical Oxygen Demand) with temperature and photoperiod as standard. All protocols were approved by the Institutional Animal Care Committee approved (CEUA–PUCRS: 7683, 2017) and followed the "Principles of Laboratory Animal Care" from the National Institutes of Health (NIH).

2.2. Gamma radiation treatment

The irradiation procedure was done similarly to that described previously (Geiger et al., 2006, 2008). In 12 well plate, 24 hpf (hours post-fertilization) embryos were gamma-irradiated with 2, 5, 10, 15 or 20 Gy using a Cobalt Theratron Phoenix (Theratronics Ltd., Ontario, Canada), in a final volume of 3 mL of system water. The plates were placed in the irradiator at a source-to-target distance of 30 cm in room temperature. The control group is representative of the mock irradiated embryos, which were also disposed in the equipment, but not exposed to radiation.

2.3. Survival curve and hatching rate

Zebrafish survival was verified daily until 7 dpf (days post fertilization), after treatments described above. It was used a minimum of 144 animals per group, performed in 3 independent experiments. Larvae position, color, locomotion and heartbeats were observed using a microscope (Nikon® SMZ 1500) to ensure animals mortality. Hatching rate was calculated as the percentage of hatched egg reported to living eggs for a given sampling time (Hu et al., 2016). The determination was performed at 72 hpf, since healthy embryos tend to leave the chorion spontaneously in the time interval of 48 to 72 hpf (Parichy et al., 2009).

2.4. Embryos heart rate

At 48 hpf, zebrafish embryos had their heartbeats monitored under the stereomicroscope (Nikon® SMZ 1500). Irradiated embryos and controls were placed in petri dishes with system water and their heartbeats were counted for 10 s and converted to heartbeat per minute (bpm) (Li et al., 2018). It was required at least 10 embryos per group, and the experiment was done in triplicate by a blind experimenter. To avoid possible interference, water temperature was kept constant at 28 °C throughout the procedure.

2.5. Morphological measurements and evaluation of pericardial edema

Evaluation of morphological changes and its measurements was performed using a stereomicroscope (Nikon® SMZ 1500) with 3× magnification (Altenhofen et al., 2017; Soares et al., 2017). The measures of body length (distance from the mouth to the pigmented tip of the tail) and surface area of the eyes were acquired through larvae photographs at 6 dpf (30 larvae per group) with the help of NSI Elements D 3.2 software, supplied by Nikon Instruments Inc. (Melville, USA). It was also determined the number of larvae that presented pericardial edema (30 larvae per group), and the rate was calculated as described by Hu et al. (2016).

2.6. Locomotor activity

The larvae locomotor activity was assessed at 6 dpf. Larvae selected from each group were transferred into a 24-well plate with one larva per well, containing 3 mL of system water at 28 °C. The recordings had 6 min, one for acclimatization and 5 for the analysis, which was performed by a tracking device (Noldus Information Technology, Wageningen, Netherlands) (Colwill and Creton, 2011). The parameters chosen to evaluate the locomotion behavior were distance (cm), velocity (cm/s) and absolute turn angle (°), using at least 12 larvae per group (Cruz et al., 2017; Capiotti et al., 2013), and the data were assessed using EthoVision XT 10.0 Software.

2.7. Enzymatic assays

2.7.1. Determination of ectonucleotidase activity

The ectonucleotidases activities were performed as described previously (Leite et al., 2013; Rico et al., 2003; Senger et al., 2004). Activities were analyzed in 6 dpf larvae, after exposure to radiation at 24 hpf. Experiments were conducted using n = 8 containing a pool of 35 larvae each. The membranes were prepared in 500 µL of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, adjusted with citric acid), centrifuged at 800 ×*g* for 15 min and the pellet was discarded. The supernatant was centrifuged at 40,000 ×*g* for 25 min. Each pellet was frozen in liquid nitrogen, thawed, suspended in 500 µL of Tris-citrate buffer and used for analysis. Zebrafish larvae membranes (3–5 µg protein) were added to the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ (for NTPDase activities) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (for ecto-5′-nucleotidase activity) in a final volume of 200 µL. All membranes were pre-incubated at 37 °C for

10 min before the initiation of the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was interrupted after 30 min by the addition of 200 μL of trichloroacetic acid 10% (final concentration 5% w/v). For the release of inorganic phosphate (Pi), samples were chilled on ice for 10 min, according to malachite green method, using KH_2PO_4 as a Pi standard (Chan et al., 1986). The non-enzymatic Pi released from nucleotides into the assay medium was subtracted from the total Pi released during the incubation, giving net values for enzyme activity. Specific activity was expressed as nmol of Pi released per minute per milligram of protein. All enzyme assays were performed at least in triplicate.

2.7.2. Determination of total adenosine deaminase activity

ADA activity was measured as described formerly (Leite et al., 2013; Rosemberg et al., 2008). Experiments were conducted using a pool of 35 larvae per group (n = 8). Larvae with 24 hpf were gamma-irradiated and at 6 dpf, and ADA activity were measured. To assess the total ADA activity, the homogenates were centrifuged at 1000 ×*g* and the supernatants were used for the activity assay. The reaction mixtures containing 50 mM sodium phosphate buffer (pH 7.0) were added to the zebrafish larvae supernatants (10 μg protein) in a final volume of 200 μL. The samples were pre-incubated for 10 min at 37 °C and adenosine was added to the medium (final concentration of 1.5 mM) to initiate the reaction. After 120 min of incubation, the reaction was interrupted by the addition of 500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml) to the samples. Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct non-enzymatic deamination of substrate. For colorimetric assay at 635 nm, the reaction medium was mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH), homogenized and incubated at 37 °C for 15 min. The ADA activity was expressed as nmol of NH_3 released per minute per milligram of protein.

2.7.3. Protein determination

Protein concentration was measured by the Coomassie blue method with bovine serum albumin as a protein standard (Bradford, 1976).

2.8. Molecular analysis by quantitative real time PCR (RT-qPCR)

The gene expression of adenosine receptors subtypes (A_1, A_{2A1}, A_{2A2} and A_{2B}), and the enzymes CD73 (Ecto-5′-nucleotidase) and ADA (ADA 1, ADA 2.1, ADA 2.2 subfamilies; ADAasi and ADA L), were determined in zebrafish larvae after gamma radiation treatment by quantitative real time PCR. At 6 dpf, zebrafish larvae were euthanized and the total RNA was isolated using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). Each sample comprises a pool of 20 zebrafish larvae each (n = 5). The quality and concentration of total RNA was estimated by A260/A280, and Deoxyribonuclease I (Invitrogen) was used to eliminate genomic DNA. Following the manufacturer's instructions, from 1 μg total RNA, the cDNA was synthesized using ImProm-II™ Reverse Transcription System (Promega). In the quantitative PCR, SYBR® Green I (Invitrogen) was used to detect double-strand DNA. Reactions were prepared in a volume of 25 μL using 12.5 μL of diluted cDNA, with a final concentration of 0.2× SYBR® Green I (Invitrogen), 100 μM dNTP, 1× PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers (Table 1). The conditions of PCR cycling were: polymerase activation for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of the cycling steps, it was included a melting-curve analysis, and fluorescence was measured from 60 to 99 °C, showing one single peak in all cases. As reference genes, EF1α and Rpl13α were used for normalization. 7500 Real-Time Systems Software v.2.0.6 (Applied Biosystems) was used to determine relative mRNA expression levels. The assays were carried out in quadruplicate (n = 4). It was also included a negative control. The efficiency per sample was calculated using LinRegPCR 2012.3 Software (http://LinRegPCR.nl) and the stability of the references genes. Relative levels of mRNA expression were determined using the $2^{-\Delta\Delta Cq}$ method (Bustin et al., 2013).

2.9. Statistical analysis

Survival curve statistical analysis was performed using the Kaplan-Meier method, considering significant a decrease of 20% of survival at the end of the evaluation. For the determination of hatching rate, heartbeats, morphological alterations and locomotor activity, the results were analyzed using one way analysis of variance (ANOVA) followed by Tukey's test. Enzymatic activities and molecular analysis were analyzed by student's *t*-test. Data are expressed as mean ± standard error, and p values < 0.05 were considered as indicative of significance in relation to control group.

3. Results

3.1. Effects of gamma radiation in zebrafish survival, hatching rate and heartbeats

Zebrafish embryos were submitted to 2, 5, 10, 15 or 20 Gy of gamma radiation at 24 hpf, and the survival assessment was performed daily up to 7 dpf. None of the groups treated showed a significant decrease in larvae survival (Fig. 1A). At 72 hpf, it was observed a decrease in the hatching rate of the animals exposed to 15 Gy and 20 Gy ($p < 0.0001$; $F_{(5,354)} = 19.34$) of radiation (Fig. 1B). All gamma ray doses (2, 5, 10, 15 or 20 Gy) caused an increase in the heartbeats per minute at 48 hpf ($p < 0.0001$; $F_{(5,451)} = 16.33$) (Fig. 1C).

3.2. Exposure to gamma rays alters zebrafish morphology and locomotor activity

We performed the evaluation of morphology and locomotion in zebrafish larvae with 6 dpf. The effect of gamma radiation in larvae morphology was assessed by the measurements of body length and ocular surface area, and by determination of the rate of animals with pericardial edema. Zebrafish exposed to 10, 15 or 20 Gy presented a significant decrease in body length ($p < 0.0001$; $F_{(5,318)} = 279.3$) and ocular surface ($p < 0.0001$; $F_{(5,318)} = 702.2$) in relation to control (Fig. 2A and B). Moreover, radiation induced a significant increase in the rate of larvae with pericardial edema at the doses of 15 Gy and 20 Gy ($p < 0.0001$; $F_{(5,138)} = 588.7$) (Fig. 2C). Interestingly, gamma rays caused changes in all parameters of locomotor activity evaluated. Groups exposed to 10, 15 or 20 Gy exhibited a decrease of distance travelled ($p < 0.0001$; $F_{(5,175)} = 65.94$) and mean velocity ($p < 0.0001$; $F_{(5,175)} = 60.70$) (Fig. 2D and E), and an increase in absolute turn angle ($p < 0.0001$; $F_{(5,175)} = 89.03$) (Fig. 2F).

3.3. Radiation disrupts adenosine metabolism and decreases A_{2B} receptor gene expression

In order to depict the adenosinergic system after radiotherapy (6 dpf), we chose the intermediate dose of 10 Gy, which was previously used as therapeutic in a zebrafish embryos xenograft tumor model (Geiger et al., 2008; Lally et al., 2007). It was determined the effect of gamma rays on ATPase, ADPase, AMPase and ADA activities, as well as their gene expression. It was also evaluated the gene expression of the 4 subtypes of P1 adenosine receptors identified in zebrafish (A_1, A_{2A1}, A_{2A2} and A_{2B}). The results showed that there were no differences in the activity of the enzymes that hydrolyze ATP and ADP, but there was a decrease in AMPase ($p = 0.0296$) and ADA activities ($p = 0.0014$) in relation to control (Fig. 3C and D). Regarding the gene expression of these enzymes, radiation increased the expression of ADA L

Table 1
Primers sequences used in RT-qPCR experiment.

Gene	Primer sequence (5′–3′)	Accession number (mRNA)	Reference
EF1α	F-CTGGAGGCCAGCTCAAACAT R-ATCAAGAAGAGTAGTACCGCTAGCATTAC	ENSDART00000023156	Tang et al. (2007)
Rpl13α	F-TCTGGAGGACTGTAAGAGGTATGC R-AGACGCACAATCTTGAGAGCAG	NM_212784	Tang et al. (2007)
A_1 (adora1)	F-GTTCCTCATTTACATTGCCATTCTGC R-TGGTTGTTATCCAGTCTCTCGCTCG	NM_001128584.1	Cruz et al. (2017)
A_{2A1} (adora2aa)	F-GCGAACTGTACGCCGAGCAGAG R-TTATTCCCAGTGAGCGGCGACTC	NM_001039815.1	Cruz et al. (2017)
A_{2A2} (adora2ab)	F-GGATTGGGTCATGTACCTGGCCATC R-GCTGTTTCCAATGGCCAGCCTG	NM_001040036.1	Cruz et al. (2017)
A_{2B} (adora2b)	F-GTTTGTTCGCTCTCTGTTGGCTGC R- CTAAAAGTGACTCTGAACTCCCGAATG	NM_001039813.2	Cruz et al. (2017)
Ecto-5′-NT	F- TGGACGGAGGAGACGGATTCACC R- GGAGCTGCTGAACTGGAAGCGTC	BC055243.1	Leite et al. (2013)
ADA 1	F- GCACAGTGAATGAGCCGGCCAC R- AATGAGGACTGTATCTGGCTTCAACG	AAH76532	Leite et al. (2013)
ADA 2.1	F- TTCAACACCACACGTATCGGGCAC R- ATCAGCACTGCAGCCGGATGATC	AAL40922	Leite et al. (2013)
ADA 2.2	F- TTGCAATTGTTCATCATCCCGTAGC R- TCCCGAATAAACTGGGATCATCG	XP_687719	Leite et al. (2013)
ADAasi	F- CTTTGTGGTACTTCAAGGACGCTTTG R- TTGTAGCAGATAAAAGAAGCGAGACG	AAL40922	Leite et al. (2013)
ADA L	F- CTCTAATGTGAAAGGTCAAACCGTGC R- AAGACGCCCTTATCATCCGTGC	NP_001028916	Leite et al. (2013)

($p = 0.0032$), an adenosine deaminase related gene (Fig. 4F). Additionally, gamma radiation had effect only in the mRNA levels of the A_{2B} receptor subtype, which presented a significant decrease in relation to the control group ($p = 0.0248$) (Fig. 5D).

4. Discussion

Gamma rays are a type of ionizing radiation commonly used in cancer radiotherapy (Baskar et al., 2012). Zebrafish in its early life stages has been shown to be a suitable model to investigate the effects of ionizing radiation (Hurem et al., 2018), and several parameters can be used as tools for a toxicological evaluation along embryo/larvae development (de Esch et al., 2012; Chakraborty et al., 2016). The aim of this study was to characterize the adenosinergic system in a zebrafish embryo radiotherapy model. Initially, to standardize the radiotherapy technique and establish the dose of radiation, we evaluated toxicological parameters after gamma irradiation. We analyzed survival, hatching rate, heartbeats, morphological changes and locomotor activity to assess the consequences of the exposure to different doses of gamma rays (2, 5, 10, 15 and 20 Gy) in 24 hpf embryos. Posteriorly, we related those findings to the modifications in adenosine metabolism and in the expression of its specific receptors.

There was no significant decrease in the animal's survival after exposure to gamma radiation at 24 hpf. In agreement to our data, previous studies suggest that embryos exposed to ionizing radiation at later stages of embryogenesis present fewer effects in larvae survival and malformations (Geiger et al., 2006; McAleer et al., 2005). In the groups gamma-irradiated with the highest doses of 15 and 20 Gy, the hatching rate was decreased, relative to a developmental delay. Interestingly, other studies reported that radiation can accelerate the hatching rate (Gagnaire et al., 2015; Pereira et al., 2011), however it was also shown that gamma radiation can slow the animal's offspring (Hu et al., 2016; Praveen Kumar et al., 2017). Zebrafish hatching succeed when embryos release the Hatching Enzyme 1 (HE1) to digest and weaken the chorion, allowing larvae spontaneous movement and its final release (De la Paz et al., 2017). Presumably, radiation can impair the secretion of the enzyme, cleave it or have an effect on larvae spontaneous movement, which consequently alters the hatching rate.

Due to its transparency, it is possible to count the heartbeat of zebrafish embryos and larvae and use this stress parameter to evaluate toxicity (Craig et al., 2006). All doses of gamma radiation tested increased the heartbeat at 48 hpf, showing that even 24 h after the exposure, the animals still presented alteration due to irradiation. Different from what we observed in this study, it has been reported that 10 Gy caused a decrease in embryos heart rate, but obtained an increase when embryos were exposed to other different radiation doses (Freeman et al., 2014). It is important to note that zebrafish is a rapidly developing animal, so there may be differences in the results depending

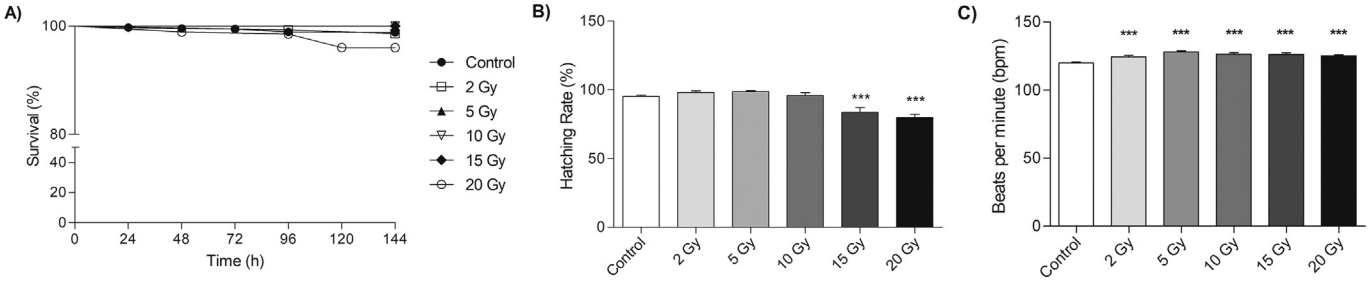

Fig. 1. Evaluation of zebrafish survival, hatching and heartbeats. (A) Embryos/larvae survival up to 7 dpf after exposure to different doses of gamma radiation at 24 hpf. Statistical analysis of the results was performed using Kaplan-Meier method, considering significant mortality above 20%. (B) Embryos hatching rate at 72 hpf. It was used at least 144 animals per group of 3 independent experiments. (C) Heartbeats of 48 hpf zebrafish embryos (minimum of 10 larvae per group, n = 3).Values are expressed as mean ± SEM. ***$p < 0.001$ indicates mean values significantly different from control group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

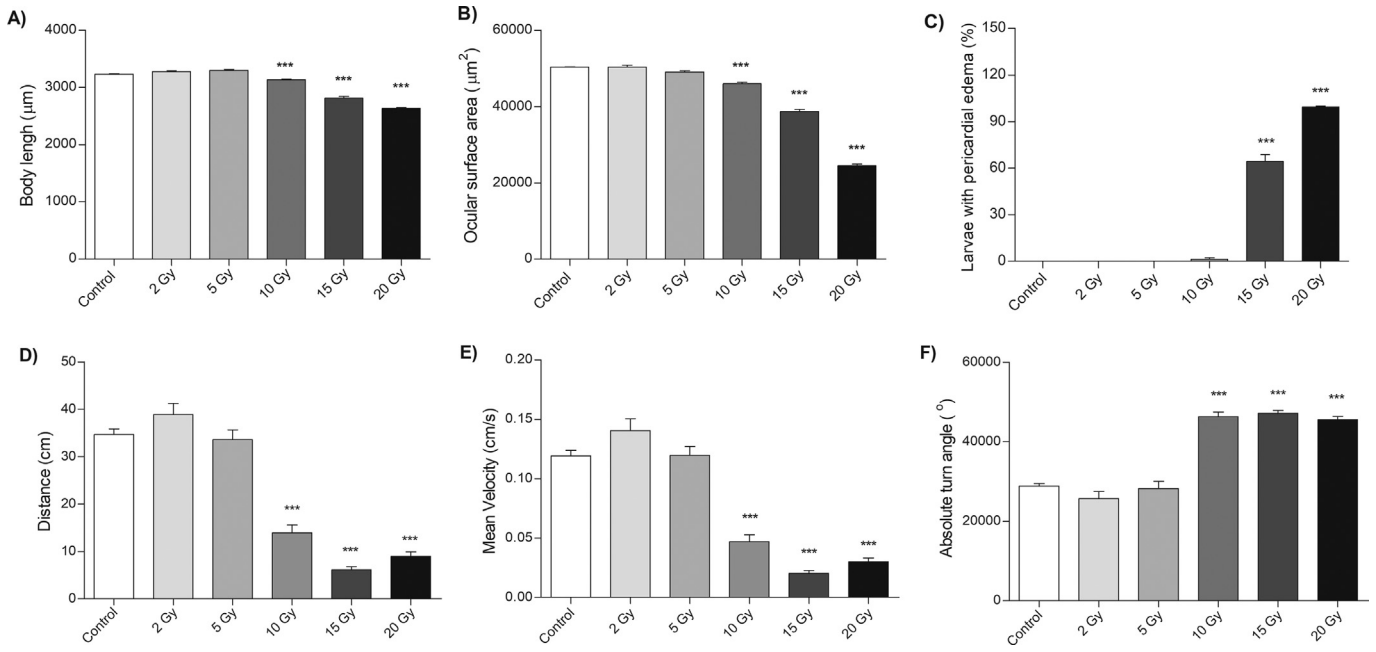

Fig. 2. Determination of morphological alterations and locomotor activity. Zebrafish embryos were exposed to gamma radiation at 24 hpf and analyzed posteriorly at 6 dpf. Deformities assessed were (A) body length, (B) ocular surface area, and (C) larvae with pericardial edema (n = 30). The parameters of locomotor activity analyzed were (D) distance, (E) mean velocity, and (F) absolute turn angle. Each bar represents the mean of at least 12 larvae per group. Data are expressed as mean ± SEM. Values significantly different from control group are indicated by ***$p < 0.001$. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

on the stage of development they were irradiated. Other factors that may influence the toxicological parameters are the dose rate and exposure duration, representative of chronic or single dose exposure.

In this study, we showed that treatments with 10, 15 and 20 Gy of gamma radiation decreased the larvae body length and ocular circumference. Other studies have reported a decrease in larvae size after irradiation at other stages of embryo development (Pereira et al., 2011; Praveen Kumar et al., 2017; Gagnaire et al., 2015). Similar to our results, the decrease in eye diameter of larvae exposed to 10 Gy was demonstrated previously (Freeman et al., 2014). Beyond that, radiation

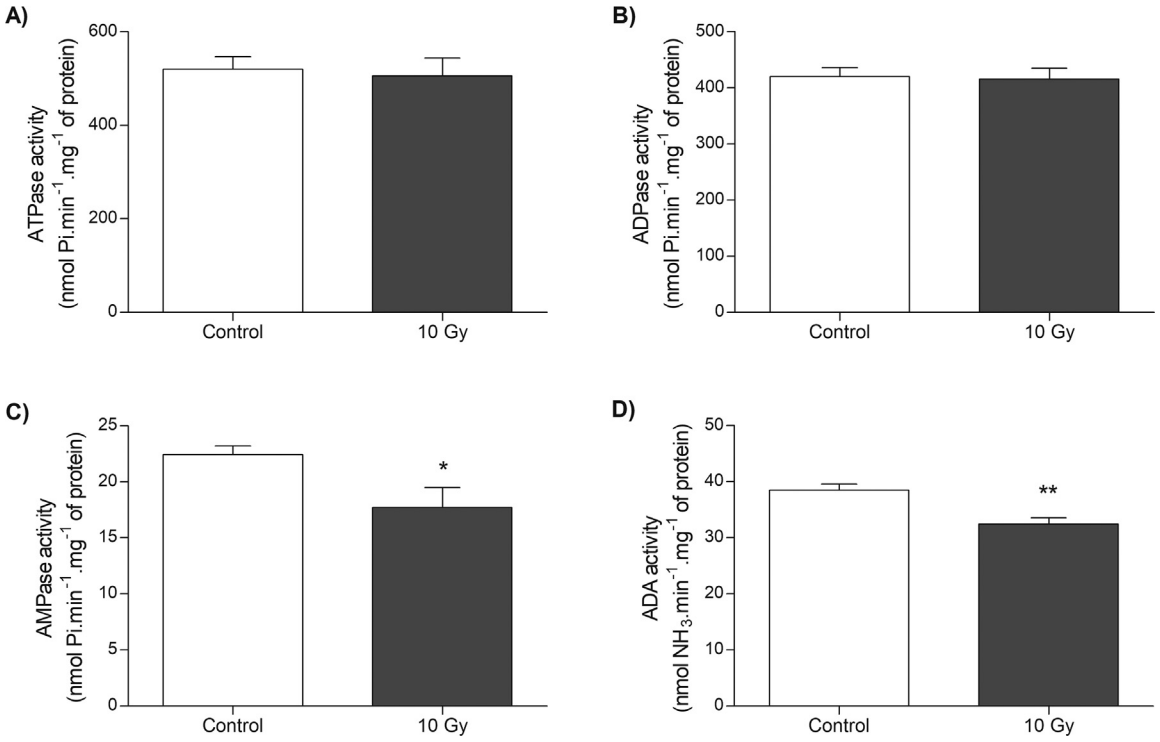

Fig. 3. Effect of 10 Gy of gamma radiation in nucleotides and adenosine hydrolysis. (A) ATPase, (B) ADPase, (C) AMPase, and (D) ADA activity (n = 8). Results are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ indicates mean values significantly different from control group. Statistical comparison of the values was assessed using Student's *t*-test analysis.

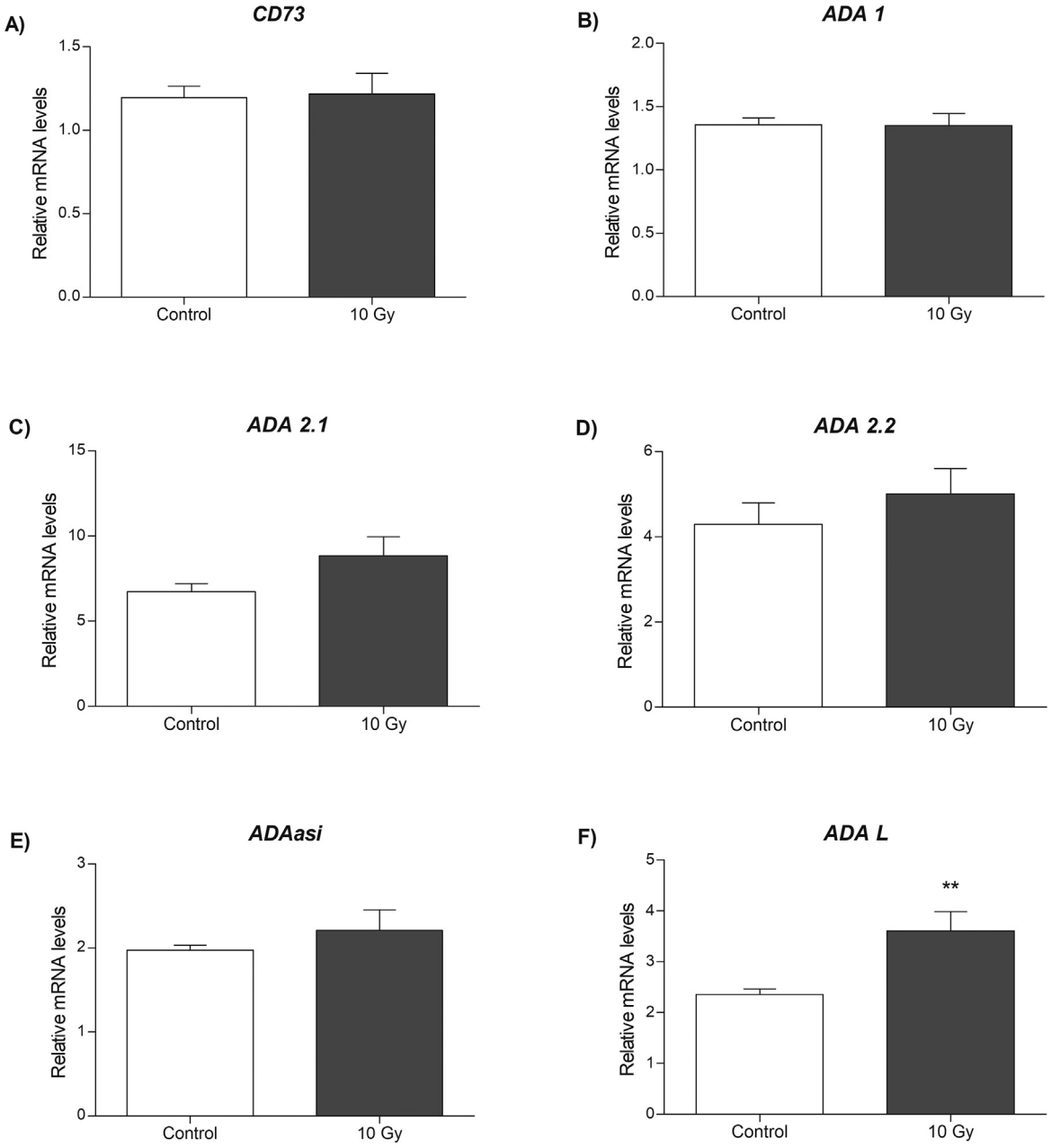

Fig. 4. Effect of exposure to 10 Gy of gamma radiation on the mRNA expression of CD73, and ADA isoforms. (A) CD73; (B) ADA 1; (C) ADA 2.1; (D) ADA 2.2; (E) ADAasi and (F) ADA L. Data are expressed as mean ± SEM (20 larvae per group, n = 4). $^{**}p < 0.01$ denotes mean values significantly different in relation to control. Results were analyzed by the Student's *t*-test.

increased the frequency of larvae malformations (Hu et al., 2016; Hurem et al., 2017; Praveen Kumar et al., 2017). The most prominent malformation seen throughout the assessments is the frequency of pericardial edema, which was significant increased in the groups exposed to 15 or 20 Gy of radiation. This finding is very interesting since radiation-induced heart diseases (RIHD), such as pericarditis, is a concern, especially in breast cancer patients who underwent radiotherapy (Rygiel, 2017).

We believe that changes in locomotor activity at the doses of 10, 15 and 20 Gy are related to the morphological alterations presented. It has been demonstrated that gamma radiation alters de structure of the muscle tissue (Gagnaire et al., 2015), body length, eyes and brain size of zebrafish larvae (Freeman et al., 2014), which can contribute to the detriment of the locomotion patterns. Although embryos exposed to 5 Gy of gamma radiation did not present changes in all the toxicological parameters that we evaluated, this dose can alter the expression of genes involved in neurological and cardiovascular functions (Freeman et al., 2014). The embryos treated with the dose of 10 Gy presented significant differences, but milder than the groups treated with the highest doses in the evaluated toxicity patterns. Moreover, 10 Gy was previously adopted to treat human tumors lineages in zebrafish embryos (Geiger et al., 2008; Lally et al., 2007). In this context, we use this irradiation procedure considered as therapeutic to characterize the adenosinergic system after radiotherapy.

CD73 is the enzyme responsible for adenosine formation through the metabolism of AMP, whereas ADA promotes the conversion of adenosine into inosine (Antonioli et al., 2013). These enzymes showed diminished activity after radiotherapy, indicating that radiation disrupts adenosine levels regulation, suggesting that lower concentrations of adenosine can exert influence in the cellular outcomes of radiation treatment. The decrease in ADA activity may be a consequence of the decrease in the formation of its substrate by CD73, in an attempt to

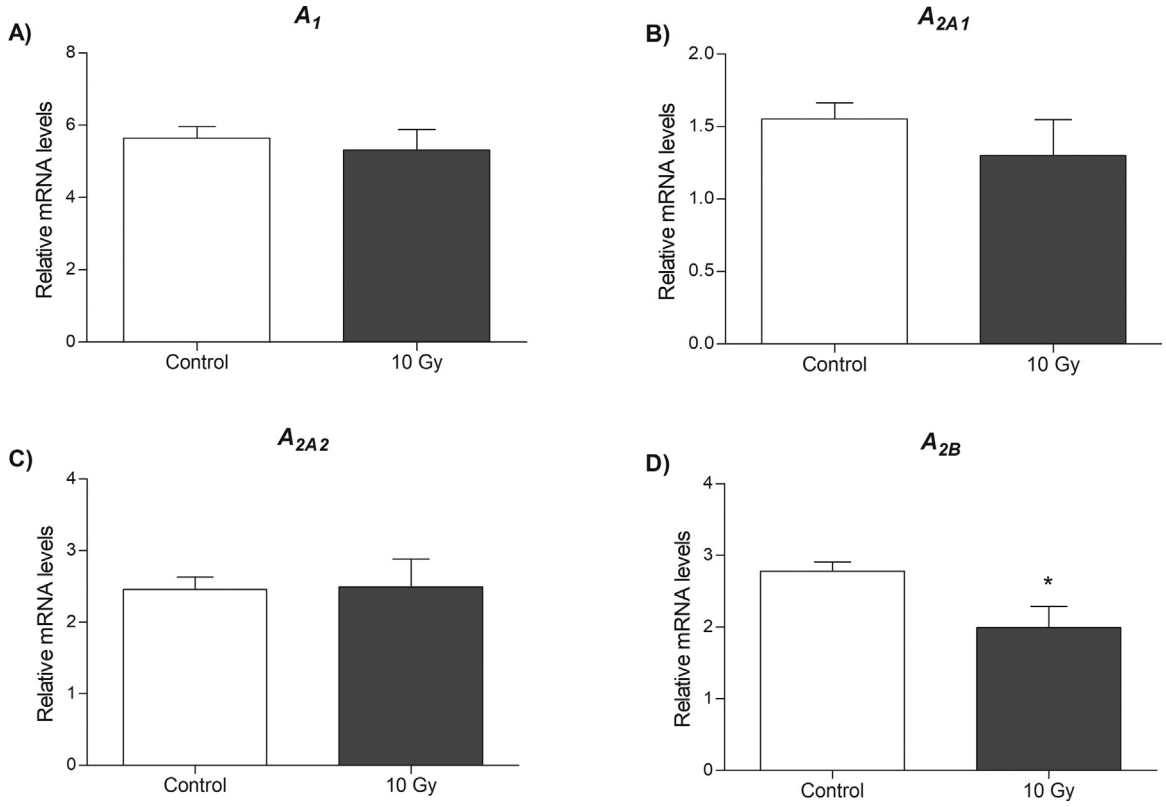

Fig. 5. Effect of exposure to 10 Gy of gamma radiation on the mRNA expression of P1 adenosine receptors subtypes. (A) A_1; (B) A_{2A1}; (C) A_{2A2} and (D) A_{2B}. Data are expressed as mean ± SEM. It was used a pool of 20 larvae per group (n = 4). $^*p < 0.05$ denotes that the mean values are significantly different to control group. Results were analyzed by the Student's *t*-test.

maintain adenosine levels. Considering that adenosine signaling is involved in the regulatory mechanisms of immunity and inflammation (Antonioli et al., 2014a; Haskó et al., 2008); and radiation toxicity is descendant of a long-term inflammatory response (Hall et al., 2016), the lower levels of adenosine may be related to the tissue damage presented by the larvae exposed to radiation. In a zebrafish model of copper-induced inflammation, the activity of the enzymes involved in adenosine metabolism was also decreased (Leite et al., 2013), reinforcing that there is an important deregulation of this nucleoside levels in inflammatory processes.

Beyond the effects associated with inflammation and cell injury, the important role of purinergic signaling in physiological fertilization, embryonic growth and developmental processes has already been revised (Burnstock and Dale, 2015). In zebrafish, the temporarily blockage of A_1, A_{2A1} and A_{2A2} adenosine receptors translation transcripts, showed the important influence of adenosine on the embryos development, which presented an increase in the rate of larvae malformation, such as eyes, tail and pericardium (Menezes et al., 2018). Since there is a disruption in adenosine levels after irradiation, these findings suggest a link between the adenosine signaling alteration with the changes in embryos/larvae development caused by gamma radiation. It is important to mention that the decrease in AMPase activity by CD73 may lead to an extracellular accumulation of AMP, which is known that can act as an adenosine receptors ligand (Jacobson and Müller, 2016). Even though there is a lack of information concerning the consequences of this activation, it is possible that some effects observed may be triggered by AMP signaling.

In this study, we used quantitative real time PCR to determine the gene expression of CD73 and ADA isoforms, which were the two enzymes that demonstrated alterations in their activity after radiation treatment. At 6 dpf, only the ADA L enzyme showed increased mRNA expression. The increase in this gene expression may be a compensatory mechanism in order to regulate the enzyme low activity. The A_{2B} receptor has a dubious role, promoting anti or pro-inflammatory effects (Antonioli et al., 2014a), however, it is known to be involved in tissue remodeling and repair processes after injury through IL-6, IL-8 and VEGF (Borea et al., 2016, 2017). Our data demonstrated that radiation decreases the gene expression of this receptor, which may have impact in the downstream intracellular pathways and immune responses triggered by its activation. In fact, the A_{2B} receptor requires high levels of extracellular adenosine to be activated (Sepúlveda et al., 2016). Therefore, in the environment generated by radiation with low concentrations of adenosine, it is possible to suggest that its gene expression may be decreased in response to the lack of the endogenous ligand.

In chronic and acute inflammatory conditions, adenosine can act as a modulator of the innate immunity (Kumar and Sharma, 2009), and it has been proposed that molecules of the immune system can be used as predictive biomarkers of radiation-induced toxicity (Sprung et al., 2015). The set of results presented here bring the possibility of investigating the components of adenosinergic system as future tools in predicting the toxic effects of radiotherapy. The role of adenosine in tumor progression has been well described, participating in the mechanisms of cell proliferation, metastasis and angiogenesis (Antonioli et al., 2014b). Other studies have already shown that tumor microenvironment presents high levels of adenosine (Young et al., 2014) and that the activation of A_{2B} receptors has major pro-tumoral actions (Gessi et al., 2011). Moreover, the blockade of CD73 enzyme has been revealed to be a promising anti-tumor strategy (Allard et al., 2016).

Our findings showed that radiotherapy promotes favorable cellular effects for the treatment of tumors through adenosine signaling. Further studies should be conducted to clarify the downstream pathways of the purinergic receptors activation that may be involved in the cellular events triggered by radiation. For the first time, the adenosinergic system was described in a zebrafish radiotherapy model, evidencing the

participation of adenosine in the tissue damage and toxic response elicited by radiotherapy. This characterization allows us to explore the adenosinergic system as a potential target in improvements of tumor radiotherapy, and in the management of radiation toxicity.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding this publication.

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