Science of the Total Environment 732 (2020) 139274

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Metformin fights against radiation-induced early developmental toxicity

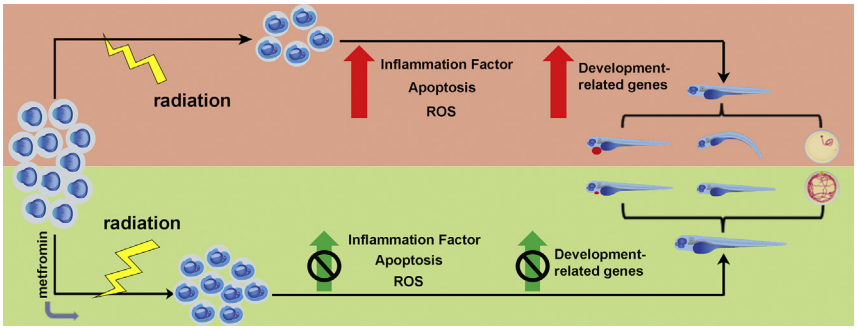
Bin Wang, Jiali Dong, Huiwen Xiao, Yuan Li, Yuxiao Jin, Ming Cui *, Shu-qin Zhang *, Sai-jun Fan *

Tianjin Key Laboratory of Radiation Medicine and Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China

HIGHLIGHTS

- Metformin mitigates radiation-disordered embryo development of aquatic organism.
- Metformin recovers body and eye size, alleviates dysmorphia and dyskinesia of irradiated zebrafish larvae.
- Metformin degrades radiation-propelled inflammatory and apoptosis response in embryos.
- Metformin reprograms development-related gene expression of embryos after radiation challenge.
- Metformin might be employed as a promising radioprotector to fight against radiation-induced early developmental toxicity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 March 2020
Received in revised form 20 April 2020
Accepted 6 May 2020
Available online 08 May 2020

Editor: Daniel Wunderlin

Keywords:
Nuclear pollution
MET
Locomotive behavior
Dysplasia
Development-related genes
Zebrafish embryo

ABSTRACT

Nuclear pollution intertwined accidental irradiation not only triggers acute and chronic radiation syndromes, but also endangers embryonic development in sight of uncontrollable gene mutation. Metformin (MET), a classic hypoglycemic drug, has been identified to possess multiple properties. In this study, we explored the radioprotective effects of MET on the developmental abnormalities and deformities induced by irradiation among three "star drugs". Specifically, zebrafish (*Danio rerio*) embryos exposed to 5.2 Gy gamma irradiation at 4 h post fertilization (hpf) showed overt developmental toxicity, including hatching delay, hatching rate decrease, developmental indexes reduction, morphological abnormalities occurrence and motor ability decline. However, MET treatment erased the radiation-induced phenotypes. In addition, MET degraded inflammatory reaction, hinders apoptosis response, and reprograms the development-related genes expression, such as sox2, sox3, sox19a and p53, in zebrafish embryos following radiation challenge. Together, our findings provide novel insights into metformin, and underpin that metformin might be employed as a promising radioprotector for radiation-induced early developmental toxicity in pre-clinical settings.

Abbreviations: MET, metformin; ROS, reactive oxygen species; IR, ionizing radiation; Gy, Gray; CON, control group; hpf, hours post fertilization; Re, resvertrol; Asp, aspirin; Et, ethanol; qRT–PCR, quantitative real-time polymerase chain reaction; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; BAX, the BCL-2-associated X protein; mTOR, mammalian target of rapamycin; AZP, 1-azakenpaullone; KEV, kevetrin hydrochloride.

* Corresponding authors.
E-mail addresses: cuiming0403@bjmu.edu.cn (M. Cui), zhangshuqin@irm-cams.ac.cn (S. Zhang), fansaijun@irm-cams.ac.cn (S. Fan).

https://doi.org/10.1016/j.scitotenv.2020.139274

1. Introduction

Disastrous incidents in nuclear facilities such as nuclear leakage at Fukushima Nuclear Power Plant and other nuclear disasters drive severe nuclear pollution (Ryuji et al., 2017). Direct exposure to ionizing radiation following radionuclides release spurs acute or chronic radiation toxicity to humans and animals (Yahyapour et al., 2018). Hematopoietic system and gastrointestinal tract are sensitive to irradiation stimuli. In addition, the casualties exposed to irradiation also represent some other complications, such as facial flushing, nausea and vomiting, and skin ulceration (Port et al., 2018). Mechanistically, ionizing radiation causes DNA fracking in different degrees directly or *via* oxidative stress, further resulting in uncontrollable genes' mutation. Animals living in radionuclide polluted regions will suffer ineluctable genetic sequelae and are susceptible to cancers (Geras'kin et al., 2008). Ionizing radiation exposure precipitates inflammation and apoptosis, and elevates reactive oxygen species (ROS) levels, which have been proved to be involved in the developmental toxicity and neurotoxicity in various models (Caballero et al., 2017; Huang et al., 2018; Xia et al., 2018). In addition, p53 and Sox family, such as sox2, sox3 and sox19a, play key roles in developmental and physiological processes of embryo, and up-regulate following radiation in zebrafish models (Okuda et al., 2010; Praveen Kumar et al., 2017). Embryo and fetus are fragile and vulnerable to radiation challenge. In detail, radiation exposure before embryo implantation elevates the rate of stillbirth overtly. In light of DNA damage and genetic variation, radiation stimuli during organogenesis stage heightens the rate of developmental abnormalities and deformities of newborns (Stepanova et al., 2016). Accidental or iatrogenic ionizing radiation exposure also precipitates irreversible genotoxicity. To date yet, radiation-induced reproductive and developmental disorders remain conundrums and starve for efficacious remedies.

Metformin (MET) has been proven to control the progression of diabetes, and become first-line hypoglycemic drug to treat type 2 diabetes worldwide during the past sixty years (Bailey and J., 2017). MET enhances insulin signaling to restrain hepatic gluconeogenesis in skeletal muscle and liver, resulting in protecting against hyperglycemia (Caballero et al., 2017). Owing to the multifunction to fight against diverse diseases, studies on MET experience a renaissance in recent years. A meta-analysis reports that MET improves the overall survival and cancer-specific survival of diabetics, implying MET might be beneficial for patients with cancers and concurrent type 2 diabetes (Yin et al., 2013). Meanwhile, mounting studies identify that MET alone or as an ancillary drug can improve the prognosis of a spectrum of cancers (Coyle et al., 2016), covering breast cancer, colon cancer, melanoma, lung cancer, pancreatic ductal adenocarcinoma, adrenocortical carcinoma and hepatocellular carcinoma *in vitro* and *in vivo* (Gerald et al., 2017; Moro et al., 2018; Paola et al., 2018; You et al., 2016). MET activates LKB1/AMPK signaling and governs stress responses, such as ROS and apoptosis, to perform anti-tumors effects (Sivalingam et al., 2014). In addition, MET impairs rapamycin complex 1 (mTORC1) binding to the target through AMPK-dependent and -independent manner, suggesting that MET might be employed as a potential agent to mitigate aging-related comorbidities and facilitate longevity (Martin-Montalvo et al., 2013). For instance, many studies have affirmed that MET can prolong the life span of different experimental models, such as flies and rats (Na et al., 2013). However, in euphoria over the successes of MET in anti-cancer and anti-aging, whether MET can be applied to fight against radiation-induced reproductive and developmental sequelae remains poorly understood.

In this study, we aimed to determine the radio-protective effects of MET on the early development of embryos and elucidate the underlying mechanism. On the basis of abundant eggs laid and rapid development of zebrafish embryos (Wang et al., 2019), we observed the developmental toxicity endpoints to unravel the possible functions of MET to embryo development, and obtained that MET protected against radiation-precipitated early developmental toxicity. This study provides evidence for metformin as a potential agent to protect against radiation-induced developmental toxicity in pre-clinical settings.

2. Materials and methods

2.1. Fish feeding and collection of embryos

Wild-type AB strain zebrafish (*Danio rerio*) were obtained from China zebrafish resource center and were sexed and maintained separately according to well established procedures on a recirculating system at 28 ± 1 °C with a 14 h light/10 h dark cycle (ShangHai HaiSheng Biotech Company, China). Fishes were fed twice daily with freshly hatched brine shrimps to ensure spawning ability. In the evening before spawning, adult fishes were segregated by sex (male/female ratio was 1/1) overnight in specially designed collection tanks. Spawning process was induced in the early morning of the day by switching on the lights. To ensure that zebrafish embryos were at the same developmental stage, embryos were collected within the 15–30 min after the barrier was removed. After fertilization, embryos were collected and washed three times with standard zebrafish Holt Buffer embryo medium (NaCl 3.5 g/L, KCl 0.05 g/L, $NaHCO_3$ 0.025 g/L, $CaCl_2$ 0.1 g/L). They were then transferred to 10 cm Petri dishes containing Holt Buffer and incubated until 3 hpf at 28.5 ± 1 °C. Finally, the embryos in each dish were observed using a dissecting microscope (Nikon, Japan), and the unfertilized eggs were removed.

2.2. Drug exposure and embryo irradiation

Fertilized embryos were transferred into a 12 well multiplate, twelve embryos per well filled with 4 mL Holt Buffer. In the section of drug selection, embryos were pretreated with resvertrol (Solarbio, Beijing, China, 0.1 mg/mL, dissolved in ethanol), aspirin (Solarbio, Beijing, China, 0.02 mg/mL, dissolved in ethanol) and metformin (Solarbio, Beijing, China, 2 mg/mL, dissolved in Holt Buffer) for 1 h before radiation exposure (5.4 Gy). We performed sensitivity tests using 0.5, 1, 2, 3, 4 mg/mL of MET for sets of zebrafish embryos at a dose of 5.2 Gy. To identify the radioprotective potential of MET with the embryonic development of zebrafish, 2 mg/mL MET was administrated at 3 hpf for 1 h (5.2 Gy). At the end of drug exposure, each set of embryos were washed three times using Holt Buffer to rinse drugs. Embryos (4 hpf) were irradiated at a dose rate of 0.88 Gy/min amounting to a total dose of 5.2 and 5.4 Gy of gamma rays (single exposure) from Gammacell" 40 Exactor (Atomic Energy of Canada Limited, Chalk River, ON, Canada) with each well containing 4 mL Holt Buffer. Once irradiation treatment was completed, the medium was renewed, and all groups were maintained at 28 ± 1 °C with a 14:10 h (light: dark) photoperiod. Holt Buffer was renewed once a day. The experiments were carried out in triplicates.

2.3. Observation of embryo development

Mortality, hatching rate, hatching process, malformations and morphological indexes of the larvae were detected as the toxicological endpoints. The embryos in the well were directly observed under a stereo microscope connected to a camera (Nikon, Japan). The survival rate was calculated as the percentage of alive embryos out of total embryos. Embryo death was defined by the disappearing of heart beat and eggs condensation. Hatching speed was recorded as the hatching rate in each treatment every 12 h up to 120 hpf. Median Hatching Time (HT50) was recorded as the time nearly for 50% of the embryos to hatch in each group. The hatching rate is calculated as the total number of embryos hatched at 120 hpf divided by the number of embryos taken for the experiment. 10 embryos with normal morphogenesis were selected randomly from each group at 48 hpf, and the heart beats in 1 min were recorded respectively using stereoscopic dissecting microscope and Media Cruiser recording software. The data for heart beats were assessed using EthoVision Heartbeat Detector software (Noldus

Information Technology, Wageningen, Netherlands). 8 normal larvae of 120 hpf from each experimental group were positioned on the lateral side using methyl cellulose, photographed and their body length, body width and eye size were measured by using software Danio Scope (Noldus Information Technology, Wageningen, The Netherlands). Deformity rates were identified by spinal curvature and pericardial edema, and the frequency of morphological deformities in embryos was calculated as the total number of larvae with morphological deformities at 120 hpf divided by the number of alive zebrafish. The proportion of each level of pericardial edema in the total pericardial edema was calculated as the total number of each deformity level at 120 hpf divided by the number of total malformations. Above measurements were carried out in triplicates.

2.4. Behavioral analysis

At 144 hpf, locomotor activity of larvae was tested to determine the influence to nervous system. Behavioral tracking was assessed with zebrafish larvae behavior analysis system at 28 °C, DanioVision (Noldus Information Technology, Wageningen, The Netherlands) and its own software EthoVision® XT. 8 larvae with normal morphogenesis were selected randomly from each group and placed into a 24 well plate flat-bottomed Falcon culture dish (BD Biosciences) at a density of one larva per well containing 300 μl Holt Buffer. The plate was placed in a temperature-controlled box with the white light and infrared camera that was connected with a computer equipped with EthoVision®XT. The larvae were allowed to acclimate to light conditions for 10 min to minimize any disturbance related handling and transporting before formal experiment. Then their locomotor activities were recorded during 20 min light-to-dark (5 min for each period and the transition lasted 20 s) transition stimulation. After tracking, the software could depict movement locus of zebrafish embryo and record distance and speed of swimming. Three replicates with 8 embryos per replicate ($n = 24$ in total) were used to assess locomotor activity.

2.5. Quantification of the expression of apoptosis and inflammation related protein

For IL-6, TNF-α, ROS, Bax and Bcl-2 measurement, 300 embryos (divided into ten groups) in each experimental group were ground up respectively with 150 μl saline, followed by centrifuging for 25 min at 3000 rpm and 4 °C. Protein level was measured from the clear supernatant using ELISA kit (Mlbio, Shanghai, China) according to the manufacturer's instructions. Optical density was read at 450 nm (Rayto, Shenzhen, China).

2.6. Gene expression

Total RNA was extracted from each group (200 zebrafish larvae divided into ten parallel groups) at 72 hpf using TRizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality and quantity of these RNA were evaluated by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer with ImPro-IIReverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to the instructions of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH Mannheim, Germany). All primers are listed in Supplementary data: Table 1. The β-actin 1 gene was selected as housekeeping gene, due to its high stability.

2.7. Statistical analysis

Each experiment was repeated at least three times. Significance was assessed by comparing the mean values (standard error of mean; SEM) using Student's *t*-test and Wilcoxon rank sum test for independent groups as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Theory/calculation

As of now, radiation-induced developmental toxicity is irreversible and intractable, starving for therapeutic avenues in clinical trials. MET, a classic drug for diabetes mellitus, has been proven as a neoteric remedy to fight against reproductive system disorder recently. Intriguingly, we identified that MET curtailed hatching process, alleviated morphological abnormalities, improved dysmorphia and dyskinesia, degraded inflammation, hindered apoptosis and reprogrammed gene expression profile of zebrafish embryos following radiation stimuli. Together, our findings support that MET may be applied to fight against radiation-induced early developmental toxicity.

4. Results

4.1. Metformin curtails radiation-delayed hatching process of zebrafish embryos

Given the multifunction of resveratrol, aspirin and metformin, we tested the safety of the three agents for embryos in a dose-dependent fashion without irradiation. Then, the radio-protective effects of the drugs on zebrafish embryos were assessed, and only MET represented radioprotection effects (Supplementary data: Figs. S1 and S2). Next, we performed sensitivity tests using 1, 2, 3 mg/mL of MET for sets of zebrafish embryos and evaluated the survival rate at day 5 after irradiation. Although survival rates were > 80% for all sets, but only 2 mg/mL set had the highest hatching rate and spinal normality rate compared to the other sets (Supplementary data: Fig. S3). Thus, the 2 mg/mL set was used as the optimal concentration to evaluate the potential protective effects for embryonic development of MET.

The hatching processes of zebrafish embryos were assessed firstly. Five Gy Gamma ray challenge reduced the embryos survival rate from 98.7% down to 94.5% at 120 h post fertilization however, MET administration improved the survival rate up to around 97% (Fig. 1A). Furthermore, irradiated embryos showed lower hatching rate (around 88% *vs* 98%) and slower hatching speed, while MET treatment heightened the hatching rate (around 97%) and accelerated the hatching speed following irradiation (Fig. 1B-D). In addition, MET also performed radioprotection to zebrafish embryos under 1 and 3 Gy irradiation (Fig. 1E-F, and Supplementary data: Fig. S4). Together, the results show that MET erases radiation-slowed hatching process.

4.2. Metformin alleviates morphological abnormalities following radiation

Morphological indexes of the larvae, such as body and eye size, were assessed following irradiation exposure with or with MET treatment at 120 hpf. As represented in Fig. 2, radiation stimuli precipitated shorter body length and narrower width, which was mitigated by MET administration (Fig. 2A-C). In parallel, MET mitigated the atrophy of eyes and slowdown of heart rate induced by radiation exposure (Fig. 2D-F). Together, our observations demonstrate that MET ameliorates morphological abnormalities following radiation challenge.

4.3. Metformin improves the dysmorphia caused by radiation

Given pericardial edema and spine bending are signs of acute radiation injury of embryo, we counted the frequency of morbidities of zebrafish larvae at 120 hpf following radiation. As shown in Fig. 3A, the normal heart rate dropped to 15% approximately after radiation exposure, while MET implement elevated the rate up to around 38%. In detail, although yolk sac edema was not appeared, the irradiated embryos showed more serious pericardial edema, represented as 40% of the embryos were concentrated in grade 2 and grade 3 (details in Fig. S4)

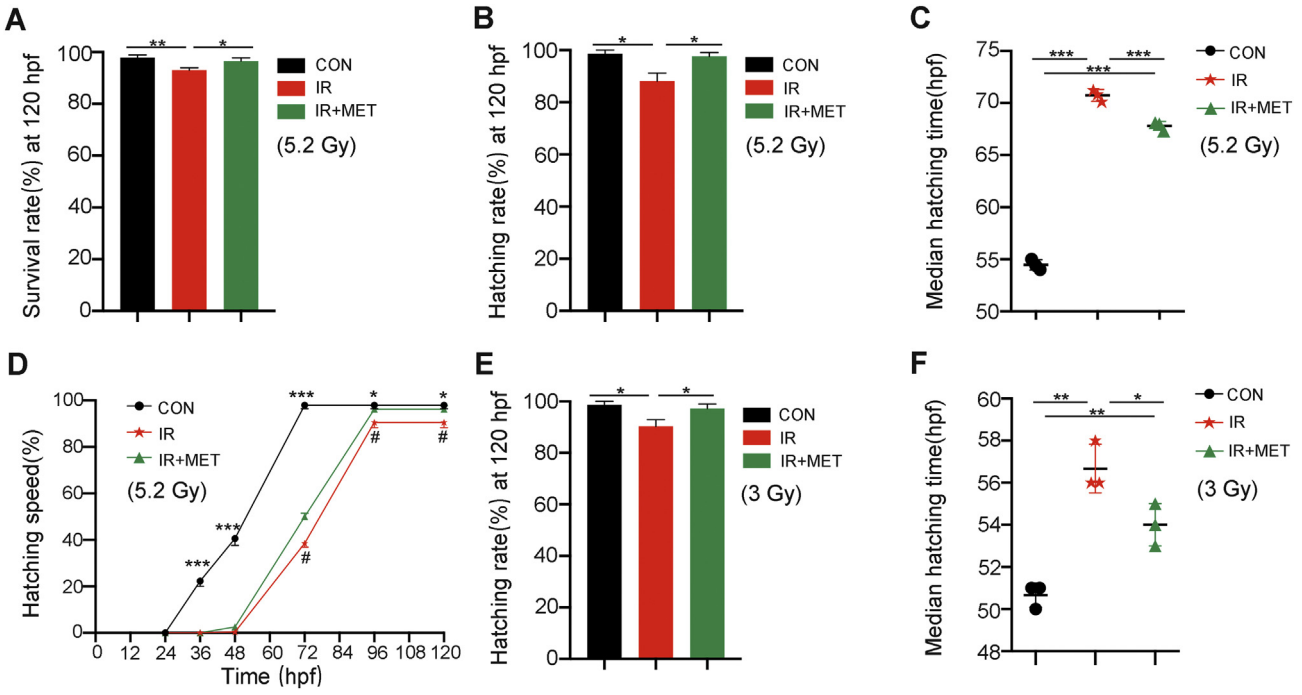


Fig. 1. MET protects against radiation-slowed hatching process. (A) Survival rate at 120 hpf following 5.2 Gy radiation. (B) Hatching rate at 120 hpf following 5.2 Gy radiation. (C) Curve of hatching speed following 5.2 Gy radiation, $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ represent Ctrl group compared with IR group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ represent MET group compared with IR group; Student's *t*-test. (D) The time spend when hatching rate reached 50% following 5.2 Gy radiation. (E) Hatching rate at 120 hpf following 3 Gy radiation. (F) The time spend when hatching rate reached 50% following 3 Gy radiation. Each experiment was repeated at least three times and each treatment group contains 108 zebrafish embryos divided into nine parallel groups. The data were presented as mean ± SEM. Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

however, MET treatment reduced the rate down to about 35% (Fig. 3B and C). In line with the observations, MET administration also increased the normal notochord rate after irradiation stimuli (Fig. 3B). Intriguingly, the irradiated embryos were all performance as grade 2 or grade 3 of spine deformities (details in Fig. S5), but the experimental embryos in MET treated cohort all stayed at grade 1 or grade 2 of spine bending (Fig. 3E and F). Together, our observations demonstrate that MET might be employed to protect against radiation-induced developmental disorders of larvae.

4.4. Metformin fights against larval dyskinesia following irradiation

Next, we tested locomotive behavior of zebrafish larvae at 144 hpf. Overall, embryos exposed to radiation showed a decrease in total

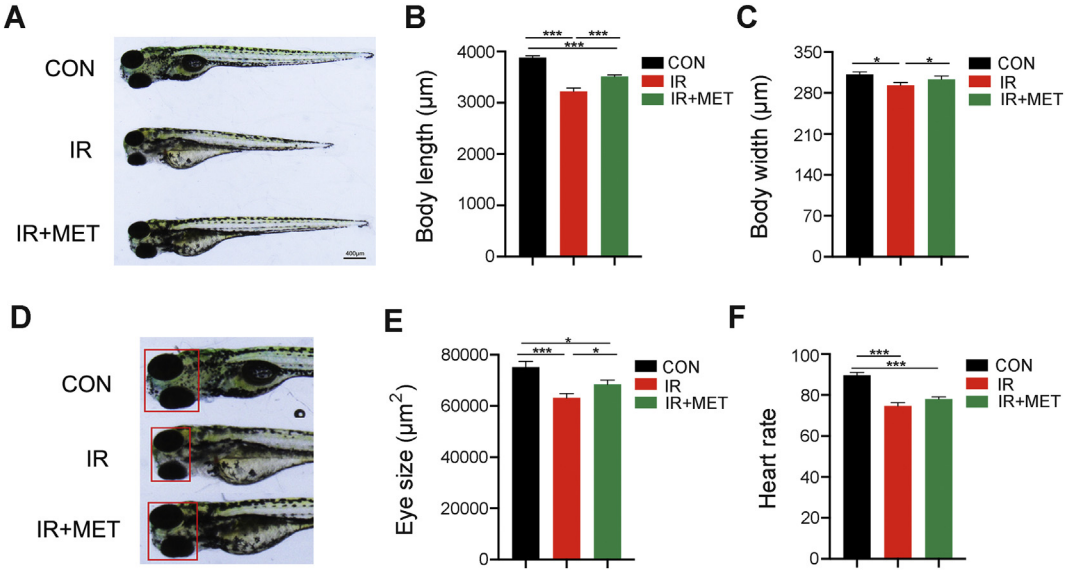


Fig. 2. MET improves the morphological indexes of the larvae following radiation challenge. (A-B) Representative stereoscopy of the effects of MET (2 mg/mL) on the body length in zebrafish larvae and corresponding statistical results. (C) Body width of zebrafish larvae at 120 hpf. (D-E) Representative stereoscopy of the effects of MET (2 mg/mL) on the eye size in zebrafish larvae and corresponding statistical results. Each experiment was repeated at least three times, $n = 8$ larvae per treatment. (F) Effects of MET (2 mg/mL) on the heart rate of zebrafish embryos after radiation exposure (5.2 Gy). The experiment was repeated at least three times, $n = 10$ embryos per treatment. All data were presented as mean ± SEM. Student's *t*-test, $^{*}P < 0.05$, $^{***}P < 0.001$.

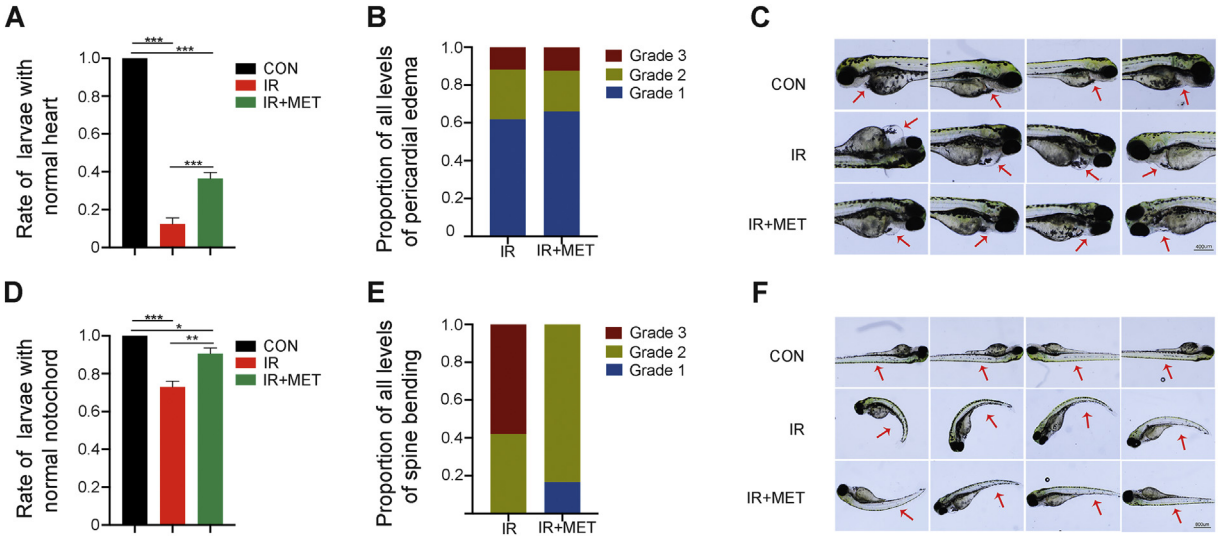


Fig. 3. MET mitigates radiation-induced deformation of zebrafish larvae. (A) Effects of MET (2 mg/mL) on the rate of larvae with normal heart after radiation exposure (5.2 Gy). (B) The proportion of each level of spine bending in the total curvature between IR and MET group. (C) Representative images of the effects of MET on the pericardial edema in zebrafish larvae. (D) Effects of MET (2 mg/mL) on the rate of larvae with normal notochord after radiation exposure (5.2 Gy). (E) The proportion of each level of pericardial edema in the total pericardial edema between IR and MET group. (F) Representative images of the effects of MET on the spine bending in zebrafish larvae. Each experiment was repeated at least three times and each treatment group contains 108 zebrafish larvae divided into nine parallel groups. The data were presented as mean ± SEM. Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

swimming distance, while MET pretreatment blocked the reduction (Fig. 4A). Specifically, analyses of motion heat map and cumulative duration of kinematic viscosity analysis also validated that larvae squinted towards more quiescence and less shifts after irradiation exposure, representing as unaltered location. However, MET administration facilitated motorial ability recovery of irradiated larvae (Fig. 4B and C). Irradiation caused more counter-clockwise rotation of larvae, but MET reversed the rotation direction (Fig. 4D). In addition, irradiated larvae also showed degressive rotation frequency and changeless movement direction during shitting in light or dark period, which were attenuated by MET treatment (Fig. 4E, F and G). Together, our observations demonstrate that MET is able to mitigate radiation-induced motor disorders of aquatic larvae.

4.5. Metformin inhibits apoptosis and inflammatory response after radiation exposure

To shed light on the underlying mechanism by which MET performs radioprotection to embryos, we assessed the apoptosis and inflammatory response following irradiation with or without MET

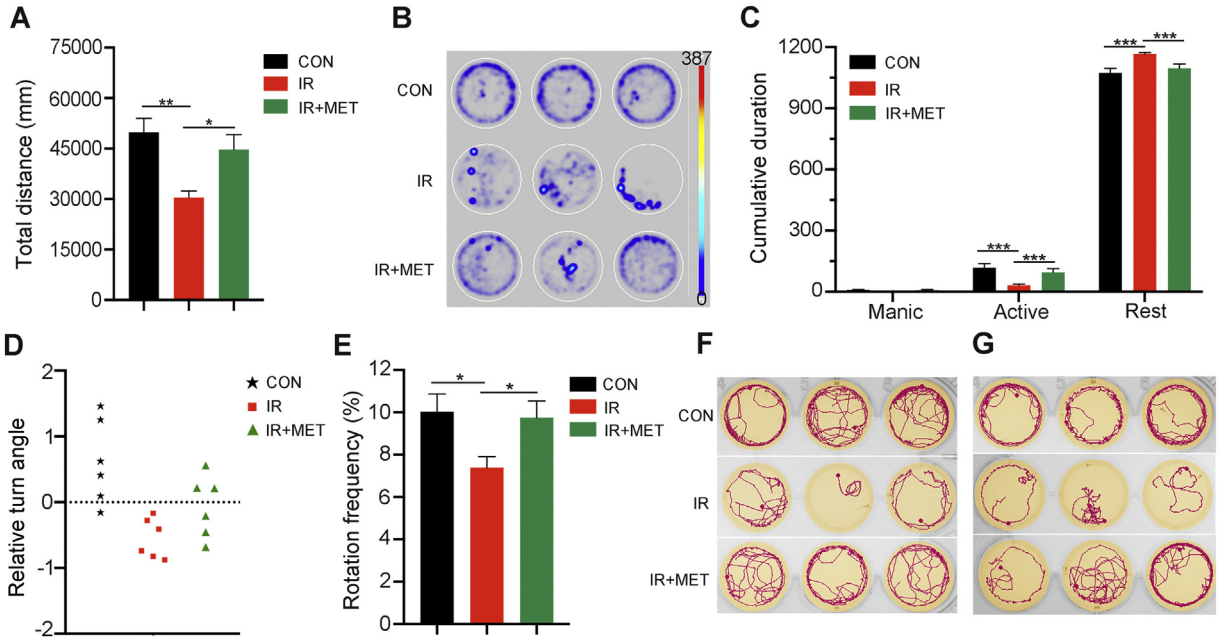


Fig. 4. MET prevents the dyskinesia of larvae from irradiation. (A) Total movement distance by light/dark alternating test. (B) Locomotion heat map of larvae recorded in 25 min. (C) Cumulative duration of different motion states by light/dark alternating test. (D) Relative turn angle by light/dark alternating test, positive value represents a clockwise direction and negative value represents a counter-clockwise direction. (E) Rotation frequency by light/dark alternating test. Take the direction of the head as the point to record data during movement. (F) Locomotion tracks of larvae in 2 min during light period. (G) Locomotion tracks of larvae in 2 min during dark period. Each experiment was repeated at least three times, $n = 6$ larvae per treatment. The data were presented as mean ± SEM. Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

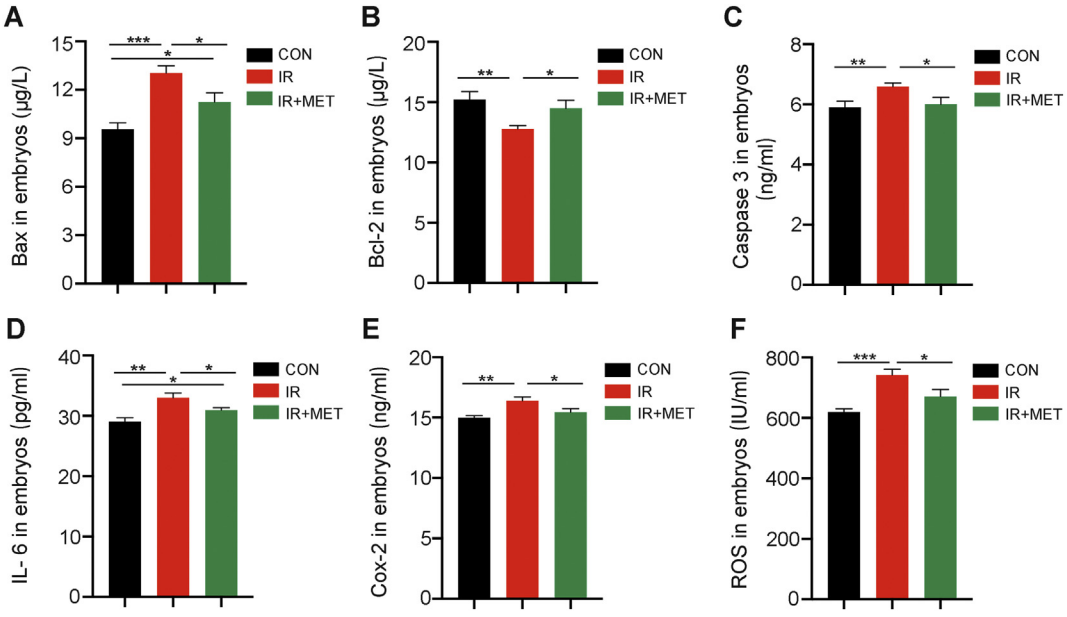


Fig. 5. MET reduces the levels of apoptosis and inflammation in zebrafish embryos at 72 hpf. (A-B) Protein levels of BAX and Bcl-2. (C) Protein levels of Caspase 3. (D-E) Protein levels of IL-6 and Cox-2. (F) Protein level of ROS. Embryos were ground up respectively and centrifuged, then the BAX, Bcl-2, Caspase 3, ROS, IL-6 or Cox-2 activity was detected by enzyme linked immunosorbent assay. Each experiment was repeated at least three times and each treatment group contains 300 zebrafish larvae divided into ten parallel groups. The data were presented as mean ± SEM. Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

treatment. As depicted in Fig. 5A-C, MET down-regulated the apoptosis promoting proteins Bax and Caspase 3, and up-regulated the restraining apoptosis proteins Bcl-2 following irradiation, indicating that MET hinders radiation-induced apoptosis of zebrafish embryos. Although the protein of TNF-α unchanged after MET administration (Fig. S6), pretreatment with MET before radiation reduced the contents of IL-6, Cox-2 and ROS (Fig. 5D-F), suggesting that MET ameliorates radiation-elicited inflammation and oxidative stress of zebrafish embryos.

4.6. MET retains the expression of developmental genes following radiation exposure

Finally, we examined the expression of key genes in early embryogenesis, such as *sox2, sox3, sox19a* and *p53*. As shown in Fig. 6A-D, radiation challenge significantly heightened the expression of *sox2, sox3, sox19a* and *p53*; however, pretreatment with MET inhibited the elevations overtly, demonstrating that MET reprograms the embryogenesis-related gene profile of zebrafish disrupted by irradiation. To verify

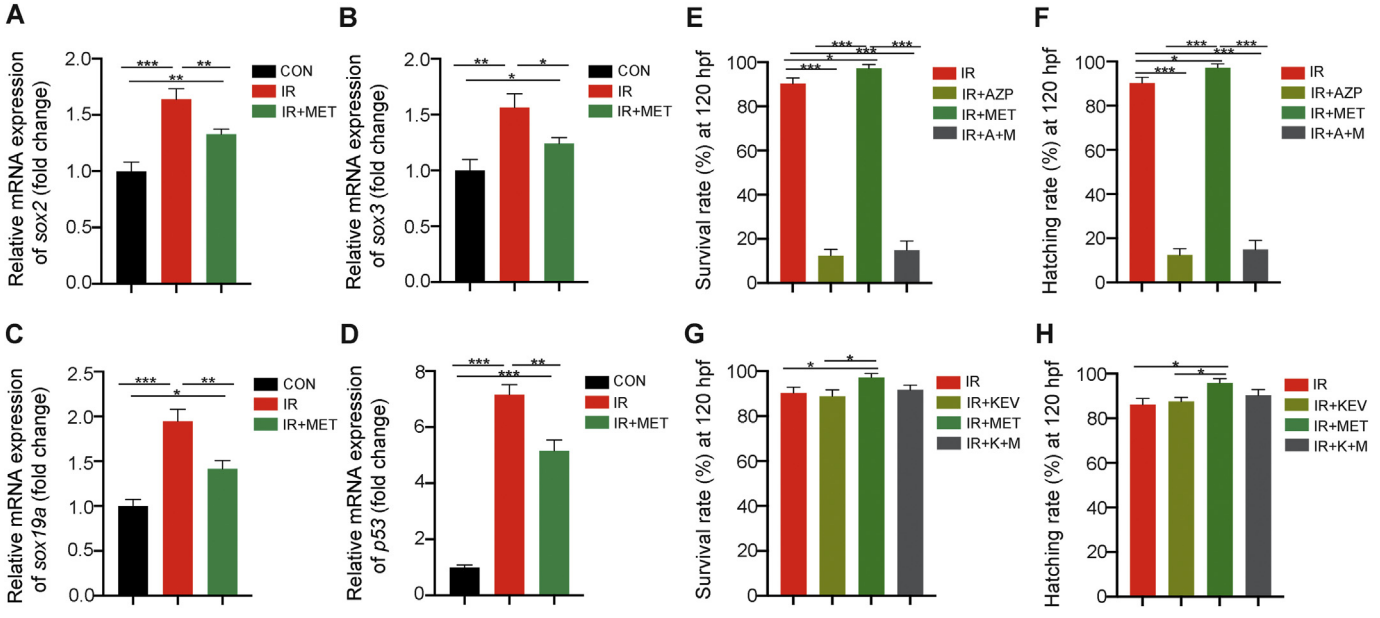


Fig. 6. MET regulates the embryogenesis-related gene profile in zebrafish embryos at 72 hpf. (A-D) Relative mRNA levels of *sox2, sox3, sox19a and p53*. Total RNA was extracted from embryos and the mRNA was detected by qRT-PCR. (*E*-F) Survival rate (E) and hatching rate (F) at 120 hpf. IR + AZP, embryos were exposed to 3 μM 1-azakenpaullone (AZP) at 2 hpf for 1 h; IR + A + M, embryos were treated with 3 μM AZP for 1 h before metformin exposure (2 mg/ml) and then accepted 5.2 Gy irradiation. (G-H) Survival rate (G) and hatching rate (H) at 120 hpf. IR + KEV, embryos were exposed to 3 μM kevetrin hydrochloride (KEV) at 2 hpf for 1 h; IR + K + M, embryos were treated with 3 μM KEV for 1 h before metformin exposure (2 mg/ml) and then accepted 5.2 Gy irradiation. Each experiment was repeated at least three times and each treatment group contains 200 zebrafish larvae divided into ten parallel groups. The data were presented as mean ± SEM. Student's *t*-test, $^{*}P < r.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

whether the radioprotection of MET depends on *Sox* family or *p53*, the embryos were exposed to corresponding agonists. Owing to no direct *Sox* family agonist was found, we used 1-azakenpaullone for activating the classic downstream signaling pathway of *Sox* family (*Wnt/β-catenin* signaling) to activate *Sox* pathway (Kormish et al., 2010) and kevetrin hydrochloride to activate *p53*. Intriguingly, agonists, especially 1-azakenpaullone, induced a serious mortality and failed hatching process following irradiation, and blocked the radioprotective function of MET (Fig. 6E-H, and Supplementary data: S7), indicating that MET fights against radiation-induced early developmental toxicity at least partly dependent on down-regulating *Sox* family and *p53*.

5. Discussion and conclusions

More than 400 nuclear power plants have been put into use around the world since 1954. Unluckily, several catastrophic nuclear incidents like accident at Chornobyl Nuclear Power Plant and Nuclear leakage at Fukushima Nuclear Power Plant happened without warning (Hasegawa et al., 2015). These disasters bring formidable harms to living things, covering human beings and animals. Radionuclides released from nuclear accidents cause tremendous hazards to marine ecological environment and reduce marine animal diversity, especially for zooplankton and fish (Buesseler et al., 2017). Enrichment of radionuclides in fish intertwines with great damage to the organs with time, which induces great impairments on the health and leads to morphological changes in their reproductive system (Lerebours et al., 2018). Ionizing radiation reprograms gene expression profile associated with DNA damage and developmental process, resulting in morphological deformities of varying degrees (Zhao et al., 2019). Although hematopoietic system is the first to fail when adult zebrafish was exposed to gamma irradiation (Traver et al., 2004), irradiation also precipitates reproductive disorders and elicits persistent genotoxicity in adult zebrafish through micronuclei accumulation and DNA damage (Hurem et al., 2018). Not surprisingly, irradiation changes the developmental traits of embryos as well, representing as smaller eyes, shorter body length, slower heart rate, decreased hatching rate (Praveen Kumar et al., 2017). Fetus are negatively influenced by ionizing radiation during pregnancy, even severe defects occurred when fetal were exposure to radiation >100 mGy (Groen et al., 2012). Relevant programs have been established for emergency response to radiation accidents (Louis et al., 2019), and some drugs for prevention and treatment of acute radiation injury have been in usage, such as potassium iodide tablets (Becker and Zanzonico, 1997). However, these drugs put on the market almost play a role in interfering with kinetic processes or absorption of radionuclides in the human body, and there are no drugs directly prevents or treats radiation-related injuries (Grebeniuk et al., 2011).

Besides relieving type 2 diabetes, MET performs identified effects in treating obesity, coronary artery disease, and other disease associated with metabolism (Tuğba et al., 2018). Interestingly, MET is used to treat some reproductive and developmental diseases, such as polycystic ovary syndrome of women with obesity or insulin resistance (Tang and T., 2006) and low ovulation rate of women at childbearing age (Naderpoor et al., 2015). What's more, MET can also be employed as a novel remedy to fight against male infertility caused by diabetes (Alves et al., 2014). Another promising exploration of MET is to figure out its different roles in radiotherapy. MET can cause oxygenation of hypoxic cells *via* downregulating oxygen consumption and cell metabolism, leading to radiosensitization in cancers (Dal Pra et al., 2013). Besides, MET inhibits tumor growth through increasing accumulation of cells in G1 or G2 phase of cell cycle, which are proved to be more sensitive to radiation (Kim et al., 2016). In addition to sensitizing tumors to radiation, MET also protects normal cells from ionizing radiation injury. An *in vivo* study shows that MET reverses pulmonary fibrosis induced by acute radiation of high dose and improves the rate of respiration in a murine model (Wang et al., 2017). In detail, the radioprotection of MET for normal tissues are dependent on scavenging of free radicals and modulating enzymes expression related to inflammation and oxidative stress (Chukwunonso Obi et al., 2016; Gómez-García et al., 2007; Shin et al., 2017). MET can also participate in DNA repair and improve genomic instability (Algire et al., 2012; Del Barco et al., 2011). Probably, the different metabolic contexts between tumor and normal cells contribute to the diverse effects of MET. Some studies show that MET has tumor targeting to a certain extent (Ohnishi et al., 2016), and MET produces more superoxide in pancreatic tumor cells by acting on mitochondria, but do not in normal cells (Cheng et al., 2016). MET inhibits mTOR signaling, which is involved in tumor hypoxia and activated in cancers. (Maity et al., 2013). Together, MET reduces cell metabolism and oxygen consumption, resulting in oxygenation of hypoxic cells and radiosensitization of cancers (Song et al., 2017). These amazing properties of MET corroborate our experimental results that MET has positive radio-protective effects on the damage of embryo development and deformities.

The ability of embryos to respond and survive after ionizing radiation changes quickly with the alteration of developmental stage. Zebrafish embryos at 2 hpf are most sensitive to radiation, but with more mortality (Honjo and Ichinohe, 2019). Importantly, the transcription of zygotic genes is initiated at blastocyst stage (around 4 hpf) (Tadros and Lipshitz, 2009). Thus, embryos at 4 hpf is a suitable experimental model to reveal the radiotoxicity and the molecular mechanisms. Given 5 Gy radiation challenge is suitable to evaluate the survival and deformity of embryos following radiation (Chai et al., 2019), we chose gamma ray radiation of 5 Gy as basic dose. Owing to the difference of radiological units and dose rates, we used 5.4 Gy in the section of drug screening, and 5.2 Gy in the phenotypic and functional studies to assess the potential protective effects of metformin. As expected, we found MET could improve the developmental toxicity induced by 5.2 Gy and lower dose radiation challenge. In addition, MET represented better radioprotective effects following 3 Gy radiation compared to 1 Gy radiation, which might be due to the slight damage at low dose and the limitation of direction method. Interestingly, our results showed MET facilitated behavioral response to light stimulation following radiation exposure as well, which was attributed to nervous system activity, including more clockwise tune angle, more rotation frequency and physical activity (Zied et al., 2018), and the changes of early neural developmental genes were also identified. Radiotherapy, as an important treatment for cancer, is necessary to control leukemia as well as malignant tumors of the head and neck, especially for central nervous system malignancies or acute lymphoblastic leukemia in children (Bindra and Wolden, 2015; Kähkönen et al., 2000). However, cranial radiation therapy has been proved to be related to well-described radiation injuries such as increased incidence of neuroendocrine impairments and neurocognitive decline (Plaga et al., 2012). Heretofore, traditional therapies for neurodegenerative diseases have yielded disappointing results. Although bevacizumab administration for the treatment of radiation brain necrosis has been proved may effective in some studies, most patients demonstrate recurrence after the discontinuation of bevacizumab (Dahl et al., 2019; Zhuang et al., 2019). In addition, the costliness of bevacizumab impedes the prospect of extended application worldwide. Thus, neurodegenerative diseases remain a conundrum and starve for efficacious remedies in clinical settings. In light of our observations, it is meaningful and interesting to further explore whether metformin has protective or therapeutic effects on radiation-induced brain injuries and other neurodegenerative diseases.

In conclusion, we firstly prove that metformin, a first-line glucose-lowering drug, improves the radiation-induced morphological abnormalities and malformation of zebrafish larvae. MET not only resists apoptosis and inflammatory response, but also regulates the expression of genes related to development following radiation challenge. Together, our findings provide novel insights into metformin, and underpin that

metformin might be employed as a potential radio-protective agent to protect against radiation-induced developmental toxicity in pre-clinical settings.

CRediT authorship contribution statement

Bin Wang: Investigation, Formal analysis, Writing - original draft. **Jiali Dong:** Investigation, Writing - original draft, Writing - review & editing.**Huiwen Xiao:** Investigation. **Yuan Li:** Writing - original draft, Writing - review & editing. **Yuxiao Jin:** Investigation. **Ming Cui:** Investigation, Formal analysis, Writing - original draft, Supervision. **Shu-qin Zhang:** Investigation, Writing - original draft, Writing - review & editing. **Sai-jun Fan:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the supports of the National Natural Science Foundation of China and Chinese Academy of Medical Science and Peking Union Medical College.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139274.

References

Algire, C., Moiseeva, O., Deschênes-Simard, X., Amrein, L., Petruccelli, L., Birman, E., et al., 2012. Metformin reduces endogenous reactive oxygen species and associated DNA damage. Cancer Prev. Res. (Phila.) 5, 536–543.

Alves, M.G., Martins, A.D., Vaz, C.V., Correia, S., Moreira, P.I., Oliveira, P.F., et al., 2014. Metformin and male reproduction: effects on Sertoli cell metabolism. Br. J. Pharmacol. 171, 1033–1042.

Bailey, J., C., 2017. Metformin: historical overview. Diabetologia 60, 1566–1576.

Becker, D.V., Zanzonico, P., 1997. Potassium iodide for thyroid blockade in a reactor accident: administrative policies that govern its use. Thyroid 7, 193–197.

Bindra, R.S., Wolden, S.L., 2015. Advances in radiation therapy in pediatric Neuro-oncology. J. Child Neurol. 31, 506.

Buesseler, K., Dai, M., Aoyama, M., Benitez-Nelson, C., Morris, P.J., 2017. Fukushima Daiichi–derived radionuclides in the ocean: transport, fate, and impacts. Annu. Rev. Mar. Sci. 9, 1.1–1.31.

Caballero, B., Olguin, N., Campos, F., Farina, M., Ballester, F., Lopez-Espinosa, M.-J., et al., 2017. Methylmercury-induced developmental toxicity is associated with oxidative stress and cofilin phosphorylation. Cellular and human studies. Neurotoxicology 59, 197–209.

Chai, Hong, Rim, Soonil, Koun, Hae-Chul, et al., 2019. Radioprotective effects of mistletoe extract in zebrafish embryos in vivo. Int. J. Radiat. Biol. 95, 1150–1159.

Cheng, G., Zielonka, J., Ouari, O., Lopez, M., McAllister, D., Boyle, K., et al., 2016. Mitochondria-targeted analogues of metformin exhibit enhanced Antiproliferative and Radiosensitizing effects in pancreatic Cancer cells. Cancer Res. 76, 3904–3915.

Chukwunonso Obi, B., Chinwuba Okoye, T., Okpashi, V.E., Nonye Igwe, C., Olisah Alumanah, E., 2016. Comparative study of the antioxidant effects of metformin, Glibenclamide, and Repaglinide in Alloxan-induced diabetic rats. J. Diabetes Res. 2016, 1635361.

Coyle, C., Cafferty, F.H., Vale, C., Langley, R.E., 2016. Metformin as an adjuvant treatment for cancer: a systematic review and meta-analysis. Annals of oncology : official journal of the European Society for Medical Oncology 27, 2184–2195.

Dahl, N.A., Liu, A.K., Foreman, N.K., Widener, M., Fenton, L.Z., Macy, M.E., 2019. Bevacizumab in the treatment of radiation injury for children with central nervous system tumors. Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery 35, 2043–2046.

Dal Pra, A., Zannella, V., Glicksman, R., Sykes, J., Muaddi, H., Joshua, A.M., et al., 2013. Metformin and prostate cancer radiation therapy: improved outcomes due to enhanced tumor oxygenation. International Journal of Radiation Oncology Biology Physics 87, S170.

Del Barco, S., Vazquez-Martin, A., Cufí, S., Oliveras-Ferraros, C., Bosch-Barrera, J., Joven, J., et al., 2011. Metformin: multi-faceted protection against cancer. Oncotarget 2, 896–917.

Gerald, D., Liubov, L., Wojciech, D., Gary, G., R, G.J., Matthew, B., et al., 2017. Metformin inhibits the development, and promotes the resensitization, of treatment-resistant breast cancer. PLoS One 12, e0187191.

Geras'kin, S.A., Fesenko, S.V., Alexakhin, R.M., 2008. Effects of non-human species irradiation after the Chernobyl NPP accident. Environ. Int. 34, 880–897.

Gómez-García, A., Martínez Torres, G., Ortega-Pierres, L.E., Rodríguez-Ayala, E., Alvarez-Aguilar, C., 2007. Rosuvastatin and metformin decrease inflammation and oxidative stress in patients with hypertension and dyslipidemia. Rev. Esp. Cardiol. 60, 1242–1249.

Grebeniuk, A.N., Zatsepin, V.V., Nazarov, V.B., Vlasenko, T.N., 2011. Modern possibilities of medicinal prophylaxis and early therapy of radiation injuries. Voen. Med. Zh. 332, 13–17.

Groen, R. S., J. Y. Bae and K. J. Lim, 2012. Fear of the unknown: ionizing radiation exposure during pregnancy. American Journal of Obstetrics & Gynecology. 206, 0–0.

Hasegawa, A., Tanigawa, K., Ohtsuru, A., Yabe, H., Maeda, M., Shigemura, J., et al., 2015. Health effects of radiation and other health problems in the aftermath of nuclear accidents, with an emphasis on Fukushima. Lancet 386, 479–488.

Honjo, Y., Ichinohe, T., 2019. Cellular responses to ionizing radiation change quickly over time during early development in zebrafish. Cell Biol. Int. 43, 516–527.

Huang, M., Jiao, J., Wang, J., Xia, Z., Zhang, Y., 2018. Characterization of acrylamide-induced oxidative stress and cardiovascular toxicity in zebrafish embryos. J. Hazard. Mater. 347, 451–460.

Hurem, S., Martín, L.M., Lindeman, L., Brede, D.A., Salbu, B., Lyche, J.L., et al., 2018. Parental exposure to gamma radiation causes progressively altered transcriptomes linked to adverse effects in zebrafish offspring. Environ. Pollut. 234, 855–863.

Kähkönen, M., Metsähonkala, L., Minn, H., Utriainen, T., Korhonen, T., Norvasuo-Heilä, M.K., et al., 2000. Cerebral glucose metabolism in survivors of childhood acute lymphoblastic leukemia. Cancer 88, 693–700.

Kim, E.H., Kim, M.S., Furusawa, Y., Uzawa, A., Sai, S., 2016. Metformin enhances the radiosensitivity of human liver cancer cells to gamma-rays and carbon ion beams. Oncotarget 7.

Kormish, J.D., Sinner, D., Zorn, A.M., 2010. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev. Dyn. 239, 56–68.

Lerebours, Adelaiede, Gudkov, Dmitri, Nagorskaya, Liubov, et al., 2018. Impact of environmental radiation on the health and reproductive status of fish from chernobyl. Environmental science & technology 52, 9442–9450.

Louis, Gauntlett, Richard, Amlt, Rubin, James, 2019. How to inform the public about protective actions in a nuclear or radiological incident: a systematic review. Lancet Psychiatry 6, 72–80.

Maity, A., Cerniglia, G.J., Daurio, N., Gallagher-Colombo, S.M., Busch, T.M., Tuttle, S.W., et al., 2013. Targeting the PI3K/mTOR pathway reduces tumor hypoxia and decreases oxygen consumption. International Journal of Radiation Oncology Biology Physics 87, S170.

Martin-Montalvo, A., Mercken, E.M., Mitchell, S.J., Palacios, H.H., Cabo, R.D., 2013. Metformin improves healthspan and lifespan in mice. Nat. Commun. 4, 2192.

Moro, M., Caiola, E., Ganzinelli, M., Zulato, E., Rulli, E., Marabese, M., et al., 2018. Metformin enhances cisplatin-induced apoptosis and prevents resistance to cisplatin in co-mutated KRAS/LKB1 NSCLC. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer 13, 1692–1704.

Na, H.J., Park, J.-S., Pyo, J.-H., Lee, S.-H., Jeon, H.-J., Kim, Y.-S., et al., 2013. Mechanism of metformin: inhibition of DNA damage and proliferative activity in Drosophila midgut stem cell. Mechanisms of Ageing & Development 134, 381–390.

Naderpoor, N., Shorakae, S., Courten, B.D., Misso, M.L., Teede, H.J., 2015. Metformin and lifestyle modification in polycysticovary syndrome: systematic review and meta-analysis. Hum. Reprod. Update 21, 560–574.

Ohnishi, S., Mizutani, H., Kawanishi, S., 2016. The enhancement of oxidative DNA damage by anti-diabetic metformin, buformin, and phenformin, via nitrogen-centered radicals. Free Radic. Res. 50, 929–937.

Okuda, Y., Kondoh, H., Kamachi, Y., 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. PLoS Genet. 344, 0–491.

Paola, S., Stefano, M., Marta, B., Francesco, M., Carla, P., Luca, R., 2018. Metformin induces apoptosis and alters cellular responses to oxidative stress in Ht29 Colon Cancer cells: preliminary findings. Int. J. Mol. Sci. 19, 1478.

Plaga, A., Shields, L.B.E., Sun, D.A., Vitaz, T.W., Spalding, A.C., 2012. Tumor histology and location predict deep nuclei toxicity: implications for late effects from focal brain irradiation. Med. Dosim. 37, 276–279.

Port, M., Pieper, B., D?rr, H.D., Hübsch, A., Majewski, M., Abend, M., 2018. Correlation of radiation dose estimates by DIC with the METREPOL hematological classes of disease severity. Radiat. Res. 189, 449–455.

Praveen Kumar, M.K., Shyama, S.K., Kashif, S., Dubey, S.K., Avelyno, D.c., Sonaye, B.H., et al., 2017. Effects of gamma radiation on the early developmental stages of Zebrafish (Danio rerio). Ecotoxicology & Environmental Safety 142, 95–101.

Ryuji, O., Kazuhiro, O., Makoto, Y.-O., Masaoki, K., 2017. A survey about the radiation effects and a health survey of Fukushima inhabitants after the Fukushima Daiichi nuclear power plant accident. J. UOEH 39, 277–290.

Shin, H.S., Ko, J., Kim, D.A., Ryu, E.S., Ryu, H.M., Park, S.H., et al., 2017. Metformin ameliorates the phenotype transition of peritoneal mesothelial cells and peritoneal fibrosis via a modulation of oxidative stress. Sci. Rep. 7, 5690.

Sivalingam, V.N., Myers, J., Nicholas, S., Balen, A.H., Crosbie, E.J., 2014. Metformin in reproductive health, pregnancy and gynaecological cancer: established and emerging indications. Hum. Reprod. Update 20, 853–868.

Song, X., Feng, L., Chao, L., Min, G., Song, G., Zhuang, L., 2017. Liposomes co-loaded with metformin and chlorin e6 modulate tumor hypoxia during enhanced photodynamic therapy. Nano Res. 10, 1200–1212.

Stepanova, Y.I., Vdovenko, V.Y., Misharina, Z.A., Kolos, V.I., Mischenko, L.P., 2016. Genetic effects in children exposed in prenatal period to ionizing radiation after the Chornobyl nuclear power plant accident. 38, 272–275.
Tadros, W., Lipshitz, H.D., 2009. The maternal-to-zygotic transition: a play in two acts. Development 136, 3033–3042.
Tang and T, 2006. The use of metformin for women with PCOS undergoing IVF treatment. Hum. Reprod. 21, 1416–1425.
Traver, D., Winzeler, A., Stern, H.M., Mayhall, E.A., Langenau, D.M., 2004. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood 104, 1298–1305.
Tuğba, Adak, Afshin, Samadi, Ayşe, Zeynep, et al., 2018. A reappraisal on metformin. Regul. Toxicol. Pharmacol. 92, 324–332.
Wang, C., Yang, L., Hu, Y., Zhu, J., Xia, R., Yu, Y., et al., 2019. Isoliquiritigenin as an antioxidant phytochemical ameliorates the developmental anomalies of zebrafish induced by 2,2′,4,4′-tetrabromodiphenyl ether. Sci. Total Environ. 666, 390–398.
Wang, J., Wang, Y., Han, J., Mei, H., Yu, D., Ding, Q., et al., 2017. Metformin attenuates radiation-induced pulmonary fibrosis in a murine model. Radiat. Res. 188, 105–113.
Xia, Q., Wei, L., Zhang, Y., Kong, H., Shi, Y., Wang, X., et al., 2018. Psoralen induces developmental toxicity in Zebrafish embryos/larvae through oxidative stress, apoptosis, and energy metabolism disorder. Front. Pharmacol. 9, 1457.
Yahyapour, R., Amini, P., Rezapour, S., Cheki, M., Rezaeyan, A., Farhood, B., et al., 2018. Radiation-induced inflammation and autoimmune diseases. Military Medical Research 5, 9.
Yin, M., Zhou, J., Gorak, E.J., Quddus, F., 2013. Metformin is associated with survival benefit in cancer patients with concurrent type 2 diabetes: a systematic review and meta-analysis. Oncologist 18, 1248–1255.
You, A., Cao, M., Guo, Z., Zuo, B., Gao, J., Zhou, H., et al., 2016. Metformin sensitizes sorafenib to inhibit postoperative recurrence and metastasis of hepatocellular carcinoma in orthotopic mouse models. J. Hematol. Oncol. 9, 20.
Zhao, W., Hu, N., Ding, D., Long, D., Li, S., Li, G., et al., 2019. Developmental toxicity and apoptosis in zebrafish embryos induced by low-dose γ-ray irradiation. Environ. Sci. Pollut. Res. 26, 3869–3881.
Zhuang, H., Shi, S., Yuan, Z., Chang, J.Y., 2019. Bevacizumab treatment for radiation brain necrosis: mechanism, efficacy and issues. Mol. Cancer 18, 21.
Zied, O., Cyril, S., Laura, C., Gilles, C., Jean-René, C., Didier, M., et al., 2018. Brainstem steering of locomotor activity in the newborn rat. J. Neurosci. 38, 7725–7740.