Protective effects of phenformin on zebrafish embryonic neurodevelopmental toxicity induced by X-ray radiation

Lu Gan\textsuperscript{a,b,c}, Menghuan Guo\textsuperscript{d,e}, Jing Si\textsuperscript{a,b,c}, Jinhua Zhang\textsuperscript{a,b,c}, Zhiyuan Liu\textsuperscript{e}, Jin Zhao\textsuperscript{f}, Fang Wang\textsuperscript{a,b,c}, Junfang Yan\textsuperscript{a,b,c}, Hongyan Li\textsuperscript{b,c}, and Hong Zhang\textsuperscript{a,b,c}

\textsuperscript{a}Department of Radiation Medicine, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China; \textsuperscript{b}Key Laboratory of Heavy Ion Radiation Biology and Medicine, Chinese Academy of Sciences, Lanzhou, China; \textsuperscript{c}University of Chinese Academy of Sciences, Beijing, China; \textsuperscript{d}School of Pharmacy, Lanzhou University, Lanzhou, China; \textsuperscript{e}College of Chemical Engineering, Northwest Minzu University, Lanzhou, China; \textsuperscript{f}Medical College of Northwest Minzu University, Lanzhou, China

ABSTRACT
Radiotherapy (RT) is a common treatment for head and neck cancers, but central nervous system function can be impaired by clinical radiation doses. This experimental study evaluated the protective efficacy of the anti-hyperglycaemic/anti-neoplastic agent phenformin against radiation-induced developmental toxicity in zebrafish embryos. Zebrafish embryos pre-treated with 25 \textmu M phenformin 1 h before x-ray irradiation were compared to irradiation-only embryos for mortality, hatching rate, morphology, spontaneous movement, heart beat, larval swimming, activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), malondialdehyde content (MDA, a by-product of membrane lipid oxidation), and acetylcholinesterase (AChE) activity. In addition, expression levels of multiple genes related to neural development and apoptosis (sod2, bdnf, ache, p53, bax, and bcl-2) were compared by RT-PCR and associated protein expression levels by western blotting. Pre-treatment with phenformin increased hatching rate, spontaneous movement, heart beat, and larval motor activity, decreased mortality and malformation rate, increased SOD, CAT, and AChE activities, and reduced MDA compared to irradiation-only embryos. The mRNA expression levels of anti-apoptotic sod2, bdnf, ache, and bcl-2 were enhanced while mRNA expression of p53 and pro-apoptotic bax were reduced in the phenformin pre-treatment group. Further, p53, Bax, and γ-H2AX (a biomarker of DNA damage) were downregulated while Bcl-2 and BDNF were upregulated by phenformin pre-treatment. Taken together, this study supports the protective efficacy of phenformin against radiation toxicity in zebrafish embryos by suppressing oxidative stress and ensuing apoptosis.

Introduction
Radiotherapy (RT) is a common treatment for head and neck cancers, nasopharyngeal cancers, primary brain tumours or brain metastases because of its ability to control cell growth. Every year, many patients with primary or metastatic brain tumours need to receive large-area or whole-brain irradiation treatment in the world [1]. However, therapeutic irradiation not only kills tumour cells but can also injure healthy neural cells, leading to cardiovascular and cerebrovascular diseases, cognitive dysfunction and myelin diseases [2–4]. Indeed, numerous reports have documented cognitive decline following cranial irradiation, including deficits in hippocampal-dependent memory function [5,6]. The damage caused by radiation is largely due to oxidative stress caused by excessive reactive oxygen species (ROS), so it is critical to develop prophylactic treatments that can mitigate irradiation-induced oxidative stress in healthy tissue without interfering with tumour eradication [7–9].

Hippocampus-dependent learning and memory are strongly dependent on neurogenesis in the dentate gyrus (DG), and radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in both animal models and human cancer patients receiving RT [10–13]. Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors regulates both hippocampal neurogenesis and the synaptotrophic mechanisms mediating hippocampal-dependent forms of learning and memory [14–18]. Irradiation exposure can reduce BDNF levels in both hippocampus and in serum [19–21]. Conversely, numerous studies have demonstrated that BDNF can protect cells against oxidative stress, inhibit apoptosis, and promote functional recovery [22,23].

The zebrafish (\textit{Danio rerio}) is a freshwater species belonging to the minnow family (Cyprinidae, order Cypriniformes) widely used as a vertebrate research model in developmental biology, toxicology, cancer research, environmental studies...
and drug development due to light transparency and general ease of maintenance and breeding. Zebrafish has also been employed extensively for studies on the developmental effects on ionizing radiation [24–27].

Phenformin is a biguanide compound with hypoglycaemic actions for type-2 diabetes similar to those of metformin. Population-based and preclinical studies have demonstrated that biguanides also possess antitumor activity both in vitro and in vivo associated with AMPK pathway modulation. Yuan et al found that phenformin is much more potent than metformin in suppressing tumour growth due to greater lipophilicity and inhibitory activity against mitochondrial complex I and cellular ATP production [28–30]. However, there is yet no evidence for radioprotective effects of biguanides.

Materials and methods

Fish maintenance and embryo collection

Adult AB wild-type strain zebrafish (D. rerio) were maintained in our laboratory (Department of Radiation Medicine, Institute of Modern Physics, Chinese Academy of Sciences) according to standard procedures. When embryos were needed for studies, one male and two female were segregated in a hatching box overnight and mated at the start of light-induced spawning. The embryos were collected and rinsed with standard zebrafish E3 culture medium (5 mmol/L NaCl, 0.33 mmol/L CaCl2, 0.33 mmol/LMgSO4.7H2O, 0.17 mmol/L KCl, pH ≈ 7.2) and incubated at 28.5°C to allow continuous development. All study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Institute of Modern Physics, Chinese Academy of Sciences. All protocols were performed in accordance with the guidelines on the Humane Treatment of Laboratory Animals stipulated by the Ministry of Science and Technology (MOST) of the People’s Republic of China.

Irradiation procedure and experimental design

Phenformin hydrochloride (Sigma, St. Louis, MO, USA), was dissolved in E3 culture medium. Pilot experiments showed that 5 – 100 μM phenformin hydrochloride exposure from 3 h post fertilisation (hpf) to 144 hpf had no obvious effect on zebrafish development. A dose of 25 μM was chosen for subsequent experiments.

Blastulas at 4 hpf with normal appearance were used for irradiation experiments. X-ray irradiation was performed using the medical accelerator at Gansu Provincial Cancer Hospital. Embryos received 4 Gy dose at a dose rate of about 0.2 Gy/min and energy of 6 MeV. Embryos were divided into four groups: (a) the control group (CK), (b) 25 μM phenformin pre-treatment group (25 μM phenformin) (c) irradiation alone group (IR), and (d) 25 μM phenformin pre-treatment + IR group receiving 25 μM phenformin treatment 1 h before X-ray irradiation.

Mortality, hatching rate and morphologic analysis

Since the embryo development is completed by 72 hpf, and the larva development is completed by 120 hpf, so, we calculated the incidence of mortality and hatching rate daily throughout the developmental time course. Mortality rate was calculated by dividing the number of death embryos at 120 hpf by the total number of embryos at the start of the trial. The hatching rate was calculated by dividing the total number of embryos hatched at 72 hpf by the number of embryos at the start of the experiment. Malformation of the larvae with abnormal development were photographed using a stereoscopic dissecting microscope (Motic, SMZ-161, Motic China Group CO., LT, China) at 30× magnification after anaesthesia with 0.01% ethyl 3-aminobenzoate methanesulphonate salt (Sigma). The malformation rates of were calculated as the percentage of malformed embryos out of the total living embryos. The per cent incidence of mortality and malformation was graphed for three replicates, each using 100 embryos.

Spontaneous movement, heart beat and larval behaviour assay

Generally, movement of the tail is considered to be spontaneous and independent of central nervous system activity as the earliest embryonic motor neurons appear at 24 hpf. Zebrafish heart differentiation is completed by 48 hpf at which time the heart beat can be clearly observed [31]. Spontaneous movement (5 min) was recorded using a stereoscopic dissecting microscope (Motic, SMZ-161) and Media Cruiser recording software (Canopus Corporation, Kobe, Japan). Data were analysed using the EthoVision XT 10.0 software (Noldus Information Technology, Wageningen, Netherlands). At 48 hpf, the zebrafish larvae were anaesthetized using 0.01% ethyl 3-aminobenzoate methanesulphonate salt (Sigma) and the heart rate (beats per minute) was calculated using a stereoscopic dissecting microscope and Media Cruiser recording software.

By 4–5 days post-fertilization (dpf), zebrafish larvae start to exhibit a range of spontaneous behaviours and a rich behavioural response to light stimulation. [32]. The development of the zebrafish nervous system was evaluated by examining swimming ability at 144 hpf as previously described [25,33–35]. Data were analysed using the EthoVision XT 10.0 software (Noldus).

Biochemical indicator tests

Superoxide dismutase (SOD) and catalase (CAT) activities, malondialdehyde (MDA) content, and acetylcholinesterase (AChE) levels were measured using commercially available kits according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).
Table 1. Primer sequences of target genes used for qRT-PCR.

| Gene name | Forward primer sequence | Reverse primer sequence |
|-----------|-------------------------|-------------------------|
| sod2      | GATAAGTCGGAGGATGCGCT   | ATGTTGACATGGCTGCTTG     |
| bdnf      | TATCAACGACAGATGGG      | GCTTCAGCATGAGAGCTCC     |
| ache      | GTGGCGACTGCGATGCT      | AGTGGCAGGGCGGAAATTACG   |
| p53       | CGGCGCGAAGATGGGACC     | CAACCGAAGACGCTGATG      |
| bax       | GCAGAATACTTACAGCGTCC   | TCCGAAATCACCAATGCTGT    |
| bcl-2     | TCACCTGTTGTCAGCCTCAT   | ACAGCCTTCACGGCAGAT      |
| β-actin   | CGAGCAGGAGATGGGACC     | CAACCGAAGACGCTGATG      |

Analysis of mRNA expression

Total RNA was extracted from zebrafish embryos at 24 hpf and quantitative real-time PCR (qRT-PCR) assays were carried out on an Applied Biosystems QuantStudio5 instrument (Thermo Fisher Scientific INC., USA) with a 20 μl reaction volume using the SYBR Premix EX Taq II kit (Takara, Japan), performed according to the manufacturer’s instructions. We detected the expression of several genes with β-actin as the house keeping gene. The primer sequences for each gene are listed in Table 1 [24,36–38].

Western blot analysis

Forty embryos in each treatment group were randomly selected for Western blot at 24 hpf. The total protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated on 10% polyacrylamide gels at 20 μg per gel lane and transferred to BVDF membranes. Membranes were blocked and labelled with antibodies against BDNF, P53, Bcl-2, Bax (all from Thermo Fisher Scientific INC., USA), γ-H2AX (Abcam, Cambridge, UK), and GAPDH (Thermo Fisher Scientific) as a gel loading control. Density values of target protein bands were measured using AlphaView software (Nature Gene Corp., Medford, NJ).

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Treatment group means were compared by one-way analysis of variance and t-tests. A p-value less than .05 or .01 (two-tailed) was considered statistically significant.

Results

Effects of phenformin on embryos hatching rate, mortality, and morphology

Zebrafish embryo mortality and malformation rates have been used to assess the toxicity or teratogenicity of numerous compounds and stressors, including radiation. To evaluate the potential protective effects of phenformin, hatchability and mortality were compared at 72 and 120 hpf, respectively, among treatment groups receiving no treatment (control), X-ray irradiation alone (IR), phenformin alone (P) or phenformin pre-treatment plus irradiation (P + IR). Compared to untreated controls, X-ray irradiation induced a significant increase in embryonic mortality (Figure 1(A), p <.01) and significant decrease in hatching rate (Figure 1(B), p <.01). X-ray irradiation also induced a variety of malformations in zebrafish embryos (Figure 1(C,D)), including tail curvature, spinal curvature, and pericardial oedema (arrow in Figure 1(D)). Pre-incubated with phenformin significantly increased the hatching rate and reduced both mortality and malformation incidence following irradiation compared to the irradiation-alone group. In contrast, pre-treatment with phenformin alone had no effect on these metrics compared to controls.

Effects of phenformin on spontaneous movement, heart rate and larval behaviour

Behavioural analysis is often used to detect the sub-lethal effects of chemicals or other external stimuli. Spontaneous curl contraction is a stereotypic locomotion mechanism during early zebrafish embryogenesis. Heart rate is a stress parameter, which can be easily measured and provides a reliable metric for developmental toxicity of a wide variety of environmental stressors. Compared to the untreated control group, embryos irradiated with x-rays exhibited a significant decrease in spontaneous movement and heart rate (p <.05) at 24 hpf and 48 hpf, respectively (Figure 2(A,B)), while phenformin pre-treatment increased locomotion and heart rate compared to the irradiation alone group.

Other behaviours also supported the protective effects of phenformin on the developing central nervous system of zebrafish. Total swimming distance in visible light decreases with incubation time in a metal substrate solution. We measured locomotor activity at 144 hpf because larvae show a richer behavioural response to light stimulation compared to younger larvae; further, larvae at this stage are mature swimmers with functioning sensory and motor systems. Total larval swimming distance in a visible-light test was significant shorter among irradiated larvae compared to the control group (p <.01), while pre-treatment with phenformin increased swimming distance compared to irradiation alone (Figure 2(C)).

Effects of phenformin on SOD activity, CAT activity, MDA content and AChE levels

Oxidative stress is a central pathogenic mechanism mediating radiation damage, suggesting that phenformin protection is associated with enhanced anti-oxidant capacity. Consistent with this notion, SOD and CAT enzyme activities were dramatically reduced by irradiation compared to the control group (Figure 3(A,B); p <.05), while accumulation of the lipid peroxidation production MDA was significantly enhanced, and all these effects were reversed by phenformin pre-treatment. In addition, AChE activity, which is highly sensitive to oxidative stress, was reduced by radiation and reversed by phenformin pre-treatment (Figure 3(C)).
Effects of phenformin on expression of cytoprotective and pro-apoptotic genes

To explore the underlying molecular mechanism of radiation-induced developmental toxicity and protection by phenformin, the mRNA expression of levels of various genes such as oxidative stress (sod2), neural development and neuroprotection (bdnf, ache), and apoptosis (p53, bax, bcl-2) were measured by RT-qPCR at 24 hpf. The expression levels of the cytoprotective genes sod2, bdnf, and bcl-2 were significantly reduced ($p < .01$) while p53, ache, and (pro-apoptotic) bax were significantly upregulated by irradiation (Figure 4). All of these changes were reversed by pre-treatment with phenformin.

Effects of phenformin on protein expression

Western blotting analysis was performed to determine the protein levels of BDNF, p53, bax, bcl-2 and γ-H2AX (a biomarker of double-stranded DNA breaks) in response to X-ray irradiation with or without phenformin (Figure 5). Levels of p53, bax and γ-H2AX were increased, while the levels of BDNF and bcl-2 were decreased by irradiation compared to the control group ($p < .01$). However, the expression of BDNF and bcl-2 had an up-regulation of and levels of p53, bax and γ-H2AX were down regulated by pre-treatment with phenformin. These are indicated that phenformin appears to protect genomic integrity under irradiation by mitigating oxidative stress and apoptosis induction.

Discussion

Zebrafish embryos are a classic model organism for evaluation of toxicity from environment factors, drugs, and various physical stressors, such as ionising radiation [35,39–41]. A large number of studies have found that both low linear energy transfer (LET) radiation, such as x-rays and γ-rays, and high LET radiation (such as carbon ion, iron ion) can induce developmental block, developmental malformations, and mortality of zebrafish embryos. Impaired heart rate, spontaneous movement, and swimming behaviour have also been reported following ionising radiation of developing zebrafish by our group and others [24,25,27,42,43]. In this study, we demonstrate that brief phenformin pre-treatment significantly reduced embryonic developmental toxicity, possibly by reducing oxidative stress, DNA damage, and apoptosis.

The mortality of irradiated embryos was significantly higher than that of the untreated group by 24 hpf, while there was no significant difference in mortality from 48 to 96 hpf. Larvae emerge from the embryo through the chorionic membrane during hatching within the latter time period (~48 – 72 hpf), indicating that the early embryos were more sensitive to ionising radiation. The decrease in survival rate following irradiation is in accord with previous reports.
Larval malformation rate was also significantly higher than that of the control group, and both mortality and malformation were significantly reduced by phenformin.

Behavioural changes during embryogenesis mirror muscular and nerve system development, and so are influenced by neuromuscular malformations and functional impairments as well as stress/emotion, learning, and memory. Spontaneous coiling is the first locomotor activity of zebrafish embryos, followed by twitching responses to touch and later by swimming. Irradiation significantly reduced both spontaneous movement prior to full CNS development (24 hpf) and swimming after motor system development (144 hpf), indicating that irradiation interfered with both muscular and motor neuron activity. Phenformin pre-treatment preserved these activities, strongly suggesting protection of both muscle and neurons. Taken together, these results indicate that phenformin has a radioprotective effect against irradiation-induced developmental toxicity.

Ionising radiation can both generate ROS and decrease antioxidant capacity, thereby creating an imbalance between oxidants and antioxidants that leads to oxidative damage. Indeed, excessive production of ROS impairs zebrafish embryonic development and alters a host of physiological and biochemical indicators. Endogenous antioxidant enzymes and exogenous antioxidants can effectively neutralise ROS and protect the organism from oxidative damage caused by ionising radiation. SOD and CAT are the first lines of defence against superoxide and hydrogen peroxide accumulation, while MDA is an end product of lipid peroxidation often measured as an index of lipid peroxidation. In the present study, irradiation dramatically reduced SOD and CAT activities, consistent with oxidative stress, while phenformin pre-treatment reversed these effects, consistent with the previous report that treatment with phenformin decreased ROS generation in caloric restriction rat [44].

AChE is a critical regulator of neuromuscular transmission and a major biomarker of oxidative stress in aquatic organisms exposed to environmental pollution [41]. Indeed, numerous toxins inhibit AChE, causing motor impairments. Oxidative stress can inhibit AChE activity and thus disrupt cholinergic neurotransmission [45]. The reduction in AChE activity following exposure to x-rays is consistent with the observed motor impairments. A previous
neurodevelopmental study found that AChE activity and expression of the neurotrophins nerve growth factor (NGF) and BDNF were altered by radiation during mouse brain development [46]. The effects of irradiation on AChE activity and BDNF expression were reversed by phenformin, providing a potential explanation for the preservation of swimming behaviour in phenformin-pre-treated irradiated larva.

ROS generation and cell apoptosis are natural events during embryogenesis [38,47]. However, excess ROS induced by
radiation can damage DNA, leading to developmental deformity and arrest. Irradiation also enhanced p53 expression. P53 is a major tumour suppressor that acts through modulation of apoptosis and the cell cycle [48,49]. Mutations in p53 have been reported due to the direct actions of ROS generated by ion irradiation, hypoxia, and chemical toxins. Induction of p53 can prevent apoptosis by promoting the expression of antioxidant genes. The protein expression levels of p53 were elevated when histone H2AX was phosphorylated and subsequently becomes γ-H2AX, a popular biomarker to evaluate the DNA damage induced by radiation [38,43]. As shown in Figure 5(A), p53 and γ-H2AX protein levels were significantly increased in zebrafish embryos exposed to irradiation in the current study, compared with control, combined with the previous biochemical indicators, these results indicate that many embryonic cells were undergoing apoptosis. Apoptosis is tightly regulated by the balance between pro-apoptotic Bax and anti-apoptotic bcl-2 expression. To further investigate the mechanism of anti-apoptotic effects of phenformin, the expression of apoptosis related mRNA and proteins were detected. Our results showed that phenformin decreased the expression of bax and enhanced the expression of bcl-2 both in mRNA and protein levels after X-ray exposure, suggesting that the mitochondrial apoptotic pathway might be involved. BDNF was demonstrated to protect the cells against oxidative attack, inhibit apoptosis, and promote the recovery function of damage neurons [23].

Figure 5. Effects of phenformin on expression of p53, bax, bcl-2 and γ-H2AX (A) and BDNF (B) in zebrafish embryos. (A,B) Assessment of p53, bax, bcl-2, γ-H2AX and BDNF in zebrafish embryos with western blot analysis. (C and D) The ratio of values of p53, bax, bcl-2, γ-H2AX and BDNF/GAPDH. Each value is expressed as the mean ± SEM. **p < .01, vs. control group; *p < .05 or **p < .05 vs. irradiation group. n = 3 replicates, 20 embryos per replicate.

Conclusion
Phenformin can significantly alleviate ROS-mediated DNA damage and lipid peroxidation in zebrafish embryos under X-ray irradiation, thereby inhibiting apoptosis, mutations, and ensuing developmental disruption and arrest. Based on these findings, further studies are warranted on the radioprotective efficacy of phenformin in mammals receiving clinical radiation doses.

Acknowledgements
We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

Disclosure statement
No potential conflict of interest was reported by the authors.
**Funding**

This work was supported by the Ministry of Science and Technology National Key R&D Project [2018YFE0205100], the National Natural Science Foundation of China [81906337, 3156254, 11605255 and 11605289], Fundamental Research Funds for the Central Universities [31920160048], and Fundamental Research Funds for the Central Universities [2017WUQ01].

**References**

[1] Cole AM, Scherwath A, Ernst G, et al. Self-reported cognitive outcomes in patients with brain metastases before and after radiotherapy. Strahlenther Onkol. 2016;192:75–76.

[2] Crossen JR, Garwood D, Glatstein E, et al. Neurobehavioral sequelae of cranial irradiation in adults – a review of radiation-induced encephalopathy. JCO. 1994;12(3):627–642.

[3] Dropcho EJ. Neurotoxicity of radiation therapy. Neurol Clin. 2010;28(1):17.

[4] Press RH, Buchwald ZS, Steuer C, et al. Report of neurotoxicity after concurrent whole brain radiation therapy and checkpoint blockade immunotherapy for patients with brain metastases. Int J Radiat Oncol. 2018;102(3):x340.

[5] Kim S, Jang BS, Jung U, et al. Gamma-irradiation is more efficient at depleting hippocampal neurogenesis than D-galactose/NaNO2. Neurosci Lett. 2011;498(1):47–51.

[6] Lee TC, Greene-Schloesser D, Payne V, et al. Chronic administration of the angiotensin-converting enzyme inhibitor, ramipril, prevents fractionated whole-brain irradiation-induced perihinal cortex-dependent cognitive impairment. Radiat Res. 2012;178(1):46–56.

[7] Huang TT, Zou Y, Corniola R. Oxidative stress and adult neurogenesis- effects of radiation and superoxide dismutase deficiency. Semin Cell Dev Biol. 2012;23(7):738–744.

[8] Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012;327(1-2):48–60.

[9] Gan L, Wang ZH, Si J, et al. Protective effect of mitochondrial-targeted antioxidant MitoQ against iron ion Fe-56 radiation induced brain injury in mice. Toxicol Appl Pharmacol. 2018;341:1–7.

[10] Ji SJ, Tian Y, Lu Y, et al. Irradiation-induced hippocampal neurogenesis impairment is associated with epigenetic regulation of bdnf gene transcription. Brain Res. 2014;1577:77–88.

[11] Monje ML, Vogel H, Masek M, et al. Impaired human hippocampal neurogenesis after treatment for central nervous system. Ann Neurol. 2007;62(5):515–520.

[12] Rola R, Raber J, Rzik A, et al. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol. 2004;188(2):316–330.

[13] Redmond KJ, Mahone EM, Terezakis S, et al. Association between radiation dose to neauronal progenitor cell niches and temporal lobes and performance on neuropsychological testing in children: a prospective study. Neuro-Oncol. 2013;15(3):360–369.

[14] Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors. 2004;22(3):123–131.

[15] Maisonnierre PC, Le Beau MM, Espinosa R, et al. Human and rat brain-derived neurotrophic factor and neurotrophin-3 – gene structures, distributions, and chromosomal localizations. Genomics. 1991;10(3):558–568.

[16] Lipsky RH, Marini AM. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann Ny Acad Sci. 2007;1122(1):130–143.

[17] Lee E, Son H. Adult hippocampal neurogenesis and related neurotrophic factors. BMB Rep. 2009;42(5):239–244.

[18] Pardone M. Role of neurotrophic factors in behavioral processes: implications for the treatment of psychiatric and neurodegenerative disorders. Vitam Horm. 2010;82:185–200.

[19] Oh SB, Park HR, Jang YJ, et al. Baicalein attenuates impaired hippocampal neurogenesis and the neocognitive deficits induced by gamma-ray radiation. Br J Pharmacol. 2013;168(2):421–431.

[20] Forbes ME, Paitsel M, Bourland JD, et al. Systemic effects of fractionated, whole-brain irradiation in young adult and aging rats. Radiat Res. 2013;180(3):326–333.

[21] Zhang YQ, Cheng ZH, Wang CL, et al. Neuroprotective effects of kojic acid against radiation-induced rat brain injury through inhibition of oxidative stress and neuronal apoptosis. Neurochem Res. 2016;41(10):2549–2558.

[22] Spina MB, Squinto SP, Miller J, et al. Selective and nonselective protective effects of brain-derived neurotrophic factor for dopaminergic-neurons in vitro – reply. J Neurochem. 1993;60(4):1582–1583.

[23] Grant MM, Barber VS, Griffiths HR. The presence of ascorbate induces expression of brain derived neurotrophic factor in SH-SYSY neuroblastoma cells after peroxide insult, which is associated with increased survival. Proteomics. 2005;5(2):534–540.

[24] Li X, Zha X, Wang Y, et al. Toxic effects and foundation of proton radiation on the early-life stage of zebrafish development. Chemosphere. 2018;200:302–312.

[25] Si J, Zhou R, Zhao BQ, et al. Effects of ionizing radiation and HLY78 on the zebrafish embryonic developmental toxicity. Toxicology. 2019;411:143–153.

[26] Wang YP, Zhou X, Zhao BQ, et al. Early embryonic exposure of ionizing radiations disrupts zebrafish pigmentation. J Cell Physiol. 2019;234(1):940–949.

[27] Zhou R, Zhang H, Wang Z, et al. The developmental toxicity and apoptosis in zebrafish eyes induced by carbon-ion irradiation. Life Sci. 2015;139:114–122.

[28] Yuan P, Ito K, Perez-Lorenzo R, et al. Phenformin enhances the therapeutic benefit of BAFV(600E) inhibition in melanoma. Proc Natl Acad Sci USA. 2013;110(45):18226–18231.

[29] Orecchioni S, Reggiani F, Talarico G, et al. The biguanides metformin and phenformin inhibit angiogenesis, local and metastatic growth of breast cancer by targeting both neoplastic and microenvironment cells. Int J Cancer. 2015;136(6):E334–E344.

[30] Miskimins WK, Ahn HJ, Kim JY, et al. Synergistic anti-cancer effect of phenformin and oxamate. PLoS One. 2014;9(11):e85576.

[31] Kimmel CB, Ballard WW, Kimmel SR, et al. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203(3):253–310.

[32] Richendrfer H, Pelkowski SD, Colwill RM, et al. On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res. 2012;228(1):99–106.

[33] Comoll RM, Creton R. Locomotor behaviors in zebrafish (Danio rerio) larvae. Behav Process. 2011;86(2):222–229.

[34] Ulhaq M, Om S, Carlsson G, et al. Locomotor behavior in zebrafish (Danio rerio) larvae exposed to perfluorooalkyl acids. Aquatic Toxicol. 2013;144:332–340.

[35] Wang ZG, Zhou R, Jiang D, et al. Toxicity of graphene quantum dots in zebrafish embryo. Biomed Environ Sci. 2015;28(5):341–351.

[36] Gagnaire B, Cavalie I, Pereira S, et al. External gamma irradiation-induced effects in early-life stages of zebrafish, Danio rerio. Aquatic Toxicol. 2015;169:122–131.

[37] Cacialli P, Danio rerio: Morpho-functional features of the gonads of zebrafish. Aquatic Toxicol. 2015;169:229–238.

[38] Danio rerio: Toxicity of graphene quantum dots in zebrafish embryo. Mutat Res-Fund Mol M. 2016;793:258–266.

[39] Wang Z, Ma J, He M, et al. Toxicity assessments of near-infrared upconversion luminescent LaF3:Yb, Er in early development of zebrafish embryos. Theranostics. 2013;3(4):351–360.

[40] Zhang ZJ, Cheang LC, Wang MW, et al. Ethanol extract of fructus Alpinia oxyphylla protects against 6-hydroxydopamine-induced damage of PC12 cells in vitro and dopaminergic neurons in zebrafish. Cell Mol Neurobiol. 2012;32(1):27–40.
[41] Parlak V. Evaluation of apoptosis, oxidative stress responses, AChE activity and body malformations in zebrafish (Danio rerio) embryos exposed to deltamethrin. Chemosphere. 2018;207:397–403.

[42] Hu M, Hu N, Ding D, et al. Developmental toxicity and oxidative stress induced by gamma irradiation in zebrafish embryos. Radiat Environ Biophys. 2016;55(4):441–450.

[43] Si J, Zhou R, Song J, et al. Toxic effects of Fe-56 ion radiation on the zebrafish (Danio rerio) embryonic development. Aquatic Toxicol. 2017;186:87–95.

[44] Anisimov VN, Ukrainsteva SV, Anikin IV, et al. Effects of phentermine and phenformin on biomarkers of aging in rats. Gerontology. 2005;51(1):19–28.

[45] Abdulwahid Arif I, Ahmad Khan H. Environmental toxins and Parkinson’s disease: putative roles of impaired electron transport chain and oxidative stress. Toxicol Ind Health. 2010;26(2):121–128.

[46] Dimberg Y, Vazquez M, Soderstrom S, et al. Effects of X-irradiation on nerve growth factor in the developing mouse brain. Toxicol Lett. 1997;90(1):35–43.

[47] Shi X, Zhou B. The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. Toxicol Sci. 2010;115(2):391–400.

[48] Lane DP. Cancer. p53, guardian of the genome. Nature. 1992;358(6381):15–16.

[49] Bates S, Vousden KH. p53 in signaling checkpoint arrest or apoptosis. Curr Opin Genet Dev. 1996;6(1):12–18.