Honokiol ameliorates radiation-induced brain injury via the activation of SIRT3

Guixiang Liao1,*, Zhihong Zhao2,*., Hongli Yang1 and Xiaming Li1

Abstract
Objective: Sirtuin 3 (SIRT3) plays a vital role in regulating oxidative stress in tissue injury. The aim of this study was to evaluate the radioprotective effects of honokiol (HKL) in a zebrafish model of radiation-induced brain injury and in HT22 cells.

Methods: The levels of reactive oxygen species (ROS), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) were evaluated in the zebrafish brain and HT22 cells. The expression levels of SIRT3 and cyclooxygenase-2 (COX-2) were measured using western blot assays and real-time polymerase chain reaction (RT-PCR).

Results: HKL treatment attenuated the levels of ROS, TNF-α, and IL-1β in both the in vivo and in vitro models of irradiation injury. Furthermore, HKL treatment increased the expression of SIRT3 and decreased the expression of COX-2. The radioprotective effects of HKL were achieved via SIRT3 activation.

Conclusions: HKL attenuated oxidative stress and pro-inflammatory responses in a SIRT3-dependent manner in radiation-induced brain injury.
Keywords
Honokiol, ionizing irradiation, reactive oxygen species, cyclooxygenase-2, sirtuin 3, radiation-induced brain injury

Date received: 16 June 2020; accepted: 15 September 2020

Introduction
An increasing number of central nervous system (CNS) and brain tumor cases have been reported, with the majority of patients receiving radiation therapy.1,2 However, radiotherapy may cause injury to tissue near the CNS. Radiation-induced brain injury may result in organ dysfunction and affect learning, memory, and cognition.3–5 There are limited effective strategies available for attenuating such injuries, and reactive oxygen species (ROS) and inflammation may play a vital role in causing radiation-induced brain injury. Accordingly, the preservation of mitochondrial function can attenuate radiation injury.6–8

The NAD$^+$-dependent deacetylase sirtuin 3 (SIRT3) is localized in the mitochondria and regulates cell metabolic homeostasis.9,10 SIRT3 is involved in mediating oxidative stress and inflammatory responses after organ injury.9,10 However, the role of SIRT3 in radiation-induced brain injury is largely unknown. Previous reports have indicated that SIRT3 activation has antioxidative and anti-inflammatory effects.11,12 In this study, we therefore used the SIRT3-activating compound honokiol (HKL) to explore the role of SIRT3 in radioprotection. HKL, also known as 2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol, is a phenolic compound isolated from Magnolia grandiflora that has various properties, including neuroprotective effects. We used a zebrafish model of radiation-induced brain injury because zebrafish are widely used in biomedical research.14

In this study, we aimed to clarify the effects of HKL and the role of SIRT3 in radiation-induced brain injury in HT22 cells and a zebrafish model, which had been established previously.15

Materials and methods

Cell culture and radiation equipment
We maintained immortalized mouse hippocampal neuronal (HT22) cells in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO$_2$, as described previously.16 The radiation equipment that was used was a 6-MV linear accelerator (Clinac 2300 EX; Varian, Palo Alto, CA, USA).

Cell viability assay and irradiation
HT22 cells were cultured in 96-well plates at a density of 5 $\times$ 10$^3$ cells/well in 200 $\mu$L complete medium. The cells were treated with or without 50 $\mu$M HKL in DMSO, 3 hours prior to exposure to 4 Gy of radiation at a dose rate of 5.0 Gy/minute. The HT22 cells were divided into four groups: control, HKL, irradiation (IR), and IR + HKL. Cell viability was evaluated 24 hours after irradiation using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) as previously described.17
**Animal experiments**

Adult (3- to 6-month-old) wild-type (AB strain) zebrafish were housed in a recirculating tank system at Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases Institute (Guangzhou, China), and were fed as previously described. The protocol was approved by the Institutional Animal Care and Use Committee at Jinan University (no. LL-KY-2019029). Zebrafish were divided into four groups \( (n = 40 \text{ per group}) \): the control group, HKL group, IR group, and IR+HKL group. Zebrafish were administered 0.15 g/L HKL in DMSO.

**Antibodies and chemicals**

The HKL, ethyl 3-aminobenzoate methane-sulfonate (MS-222), and antibodies against SIRT3 and cyclooxygenase-2 (COX-2) were obtained from Sigma-Aldrich. The antibody against \( \beta \)-actin was purchased from Cell Signaling Technology (Beverly, MA, USA). The kits for evaluating ROS, interleukin-1 beta (IL-1\( \beta \)), and tumor necrosis factor-alpha (TNF-\( \alpha \)) were obtained from Nanjing Bioengineering Institute (Nanjing, China).

**Zebrafish irradiation**

Irradiation of zebrafish brains was performed as previously described. Briefly, zebrafish were administered HKL 3 hours prior to the radiation exposure. They were anesthetized by immersion in 0.02% MS-222, and were then exposed to a single dose (20 Gy) of cranial radiation, which is a sublethal dose for an adult zebrafish. Irradiation was delivered at a dose rate of 5.0 Gy/minute at a distance of 100 cm from the source to the axis.

**Dissection of the zebrafish hippocampus**

Before dissection, the zebrafish were anesthetized using 0.02% MS-222. Subsequently, the zebrafish were euthanized by immersion in an ice-water bath for 5 minutes. Hippocampus dissection was performed as previously described.

**Biochemical assays**

HT22 cells and zebrafish hippocampi were homogenized in Tris-HCl buffer (pH 7.4) and centrifuged. The supernatants were collected for biochemical analysis. ROS were measured using 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) staining, 24 hours after irradiation. Staining was performed according to the manufacturer’s instructions and as previously described. The TNF-\( \alpha \) and IL-1\( \beta \) levels were detected using an enzyme-linked immunosorbent assay (ELISA) at 24 hours after HT22 cell and zebrafish hippocampal tissue irradiation. Hippocampal tissue and HT22 cells were homogenized in 1 mL of ice-cold phosphate-buffered saline. After three freeze–thaw cycles, the homogenates were centrifuged for 5 minutes at 10,000 \( \times \) g at 4°C. Protein concentrations were measured using a bicinchoninic acid reagent. The IL-1\( \beta \) and TNF-\( \alpha \) levels were measured using commercial ELISA kits as per the manufacturer’s instructions.

**RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (PCR) amplification**

Total RNA was extracted from zebrafish hippocampi and HT22 cells using TRIzol reagent (Takara, Dalian, China) according to the manufacturer’s instructions. RNA concentrations were measured, and cDNA synthesis and quantitative real-time PCR were performed as previously described. The primer sequences are shown in Table 1.
Transfection with short interfering RNA (siRNA)

The SIRT3 siRNA was designed and manufactured by RiboBio Co. (Guangzhou, China). HT22 cells were transfected using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. Experiments using transfected cells were performed 48 hours after transfection. Scrambled siRNA was used as a control.

Western blot assays

Proteins were extracted from zebrafish hippocampi and HT22 cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology) containing a phosphatase inhibitor cocktail and proteinase inhibitor cocktail (Sigma-Aldrich), according to previously described methods. The protein concentrations were detected and western blot assays were performed as previously described. Each experiment was independently performed at least three times.

Statistical analysis

The data analyses were conducted using SPSS for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and the least significant difference test were used. A P-value less than 0.05 was considered statistically significant.

Results

HKL increases HT22 cell viability after radiotherapy and decreases pro-inflammatory cytokine and ROS levels

The MTT assay, performed 24 hours after cell irradiation, revealed that HKL significantly increased cell viability in the IR+HKL group compared with the IR group (P < 0.05) (Figure 1(a)). Conversely, HKL treatment significantly decreased levels of the pro-inflammatory factors TNF-α, IL-1β (Figure 1(b,c)), and ROS (Figure 1(d)) compared with the RT group (P < 0.05).

HKL decreases COX-2 expression and increases SIRT3 expression in HT22 cells

The COX-2 and SIRT3 levels were measured in HT22 cells 24 hours after irradiation. As shown in Figure 2(a,b), HKL treatment significantly decreased COX-2 mRNA levels and increased SIRT3 mRNA levels in HT22 cells in the IR+HKL group compared with the IR group (P < 0.05). Moreover, HKL treatment significantly decreased COX-2 protein levels and increased SIRT3 protein levels in the IR+HKL group compared with the IR group (P < 0.05; Figure 2(c)).

HKL attenuates radiation-induced injury via SIRT3 activation in HT22 cells

The HT22 cells were transiently transfected with SIRT3 siRNA. The effects of this

Table 1. The primer sequences used to amplify genes in the zebrafish and in HT22 cells.

| Gene | Zebrafish | HT22 cells |
|------|-----------|------------|
| β-actin | F: GTGCCCATCTACGAGGGTTA  
R: TCTCAGCTGTTGGTGTAGAG | F: AGCCATGTACGAGCCATCC  
R: CTCTAGCTGTTGGTGTAGAG |
| SIRT3 | F: CATTAATGGTGGATAAGGCCC  
R: AGTTCCCTCTCCTTTGTAATCCCTCGGAC | F: ATCCGGACTCTCAAGTCGCC  
R: CAACAGTAAAGAGGCTTGG |
| COX-2 | F: TATTGGAGAGCGCTGGGAGTTCA  
R: CAAATTCTCCTCTTCCGGGAT | F: ATCTGGGTTGCGGAGCACAA  
R: GTGGTAAAGCGCTCAGGTGTT |

COX-2, cyclooxygenase-2; F, forward; R, reverse; SIRT3, sirtuin 3.
siRNA were assessed using western blot. As presented in Figure 3(a), SIRT3 siRNA effectively inhibited the expression of SIRT3. The role of SIRT3 signaling in the radioprotective effects of HKL was evaluated in HT22 cells after irradiation. Cell survival was evaluated using the MTT assay. As shown in Figure 3(b), radiation led to reduced cell viability in SIRT3-deficient cells compared with control cells. Furthermore, HKL did not significantly increase the survival of SIRT3-deficient cells (Figure 3(b)). The levels of ROS were significantly increased in the SIRT3-deficient cells compared with the control cells (Figure 3(c)). Furthermore, HKL treatment did not reduce the levels of ROS in irradiated SIRT3-deficient cells.

**HKL decreases pro-inflammatory responses and ROS levels in the zebrafish hippocampus**

As presented in Figure 4(a,b), the TNF-α and IL-1β levels were significantly decreased in the IR+HKL group compared with the IR group ($P < 0.05$). Similarly, the levels of ROS were decreased in the IR+HKL group compared with the IR group ($P < 0.05$; Figure 4(c)).
HKL increases SIRT3 expression and decreases COX-2 expression in the zebrafish hippocampus

Both the mRNA and protein levels of SIRT3 and COX-2 were measured in the zebrafish hippocampus. HKL significantly reduced the mRNA and protein expression of COX-2 in the IR+HKL group compared with the IR group ($P < 0.05$; Figure 4(d,f)). Furthermore, the mRNA and protein expression of SIRT3 was significantly increased in the IR+HKL group compared with the IR group ($P < 0.05$; Figure 4(e,f)).

Discussion

Radiation-induced brain injury is a common occurrence in patients who have received radiotherapy for head, neck, or brain tumors. Inflammation and oxidative stress are the major mechanisms involved in radiation-induced brain injury. However, the exact mechanisms are not yet fully known. Radiation-induced ROS production contributes to tissue damage and oxidative DNA damage. Moreover, the generation of free radicals may also activate TNF-$\alpha$ and IL-1$\beta$ and upregulate pro-inflammatory pathways.

Some antioxidants have radioprotective roles. For example, HKL has been demonstrated to have antioxidative activity and neuroprotective effects in several CNS diseases. In the present study, increased ROS production was observed in the hippocampus of zebrafish after irradiation, which corroborated the results of previous reports.
Persistent ROS generation contributes to brain damage and dysfunction.\textsuperscript{32}

HKL is a major bioactive constituent of the Chinese medicinal plant \textit{M. officinalis}. In HKL, the hydroxyl group of the second phenol possesses good chemical reactivity with peroxyl radicals.\textsuperscript{33} HKL suppresses mitochondrial complex I-dependent respiration, stimulates the formation of mitochondrial ROS, induces 5\textsuperscript{th} adenosine monophosphate-activated protein kinase activation, and inhibits mitochondrial signal transducer and activator of transcription phosphorylation. Notably, the inhibition of mitochondrial complex I activity and subsequent increase in ROS formation has been proposed as a key factor in the chemoprevention and antitumor mechanisms of HKL.\textsuperscript{34}

The present study indicated that HKL treatment significantly inhibits ROS generation in irradiated HT22 cells as well as the zebrafish brain. HKL typically ameliorates oxidative stress and inflammation in brain cells.\textsuperscript{35–37} Our study also demonstrated that HKL can attenuate not only ROS levels, but also TNF-\textalpha{} and IL-1\textbeta{} levels.

SIRT3 is involved in the antioxidant pathway and is associated with several human diseases.\textsuperscript{38} Our results indicated that HKL treatment activates SIRT3 expression at both the mRNA and protein levels, and that it also reduces the expression of COX-2 and pro-inflammatory cytokines, thus mitigating radiation-induced brain injury. These findings are in line with previous studies that revealed an inhibition of inflammatory responses upon SIRT3 activation. In HT22 cells, we also observed that HKL treatment attenuated radiation-induced injury via SIRT3 activation. Moreover, SIRT3 activation may have protective roles in other cell types.\textsuperscript{39} For example, Cao et al. reported that SIRT3
activation can alleviate radiation-induced lung injury.\textsuperscript{40} HKL did not have a radioprotective role in SIRT3-deficient cells, suggesting that SIRT3 is involved in oxidative damage. Moreover, studies have reported that SIRT3 deficiency abrogates the radioprotective effects of SIRT3 activator\textsuperscript{11,29}. Additionally, SIRT3-knockout mice have exaggerated cardiac dysfunction during ischemia–reperfusion.\textsuperscript{41} A SIRT3 activator compound also failed to demonstrate a protective role in SIRT3-knockout mice with acute lung injury.\textsuperscript{12}

In conclusion, our study indicates that HKL treatment has radioprotective effects via the activation of SIRT3, which in turn attenuates oxidative stress injury and pro-inflammatory responses.

**Acknowledgements**

We would like to thank Editage (www.editage.com) for English language editing. We also
thank Baiyao Wang and Yawei Yuan for their help.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

Funding
The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Shenzhen Health and Family Planning System Research (no. SZBC2017024), the Natural Science Foundation of Shenzhen (project number: JCYJ20170307095828424), and Cultivation Research for the Youth of Shenzhen People’s Hospital (no. SYKYPY2019029).

ORCID iD
Guixiang Liao https://orcid.org/0000-0002-4369-8811

References
1. Siegel R, Ma J, Zou Z, et al. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9–29.
2. Ramanan S, Kooshki M, Zhao W, et al. The PPARalpha agonist fenofibrate preserves hippocampal neurogenesis and inhibits microglial activation after whole-brain irradiation. Int J Radiat Oncol Biol Phys 2009; 75: 870–877.
3. Prasanna PG, Ahmed MM, Stone HB, et al. Radiation-induced brain damage, impact of Michael Robbins’ work and the need for predictive biomarkers. Int J Radiat Biol 2014; 90: 742–752.
4. Armstrong GT, Liu Q, Yasui Y, et al. Long-term outcomes among adult survivors of childhood central nervous system malignancies in the Childhood Cancer Survivor Study. J Natl Cancer Inst 2009; 101: 946–958.
5. Gondi V, Pugh SL, Tome WA, et al. Preservation of memory with conformal avoidance of the hippocampal neural stem-cell compartment during whole-brain radiotherapy for brain metastases (RTOG 0933): a phase II multi-institutional trial. J Clin Oncol 2014; 32: 3810–3816.
6. Liu T, Pei H, Xu D, et al. GANRA-5 protects mice from X-ray irradiation-induced dysfunction of the immune system. Free Radic Res 2014; 48: 875–882.
7. Greenberger JS and Epperly MW. Antioxidant gene therapeutic approaches to normal tissue radioprotection and tumor radiosensitization, In Vivo 2007; 21: 141–146.
8. Li P, Zhao QL, Wu LH, et al. Isofraxidin, a potent reactive oxygen species (ROS) scavenger, protects human leukemia cells from radiation-induced apoptosis via ROS/mitochondria pathway in p53-independent manner. Apoptosis 2014; 19: 1043–1053.
9. Liu J, Li D, Zhang T, et al. SIRT3 protects hepatocytes from oxidative injury by enhancing ROS scavenging and mitochondrial integrity. Cell Death Dis 2017; 8: e3158.
10. Lombard DB, Alt FW, Cheng HL, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol Cell Biol 2007; 27: 8807–8814.
11. Zhao W, Zhang L, Chen R, et al. SIRT3 protects against acute kidney injury via AMPK/mTOR-regulated autophagy. Front Physiol 2018; 9: 1526.
12. Kurundkar D, Kurundkar AR, Bone NB, et al. SIRT3 diminishes inflammation and mitigates endotoxin-induced acute lung injury. JCI Insight 2019; 4: e120722.
13. Ye JS, Chen L, Lu YY, et al. Honokiol-mediated mitophagy ameliorates postoperative cognitive impairment induced by surgery/sevoflurane via inhibiting the activation of NLRP3 inflammasome in the hippocampus. Oxid Med Cell Longev 2019; 2019: 8639618.
14. Won SY, Choi BO, Chung KW, et al. Zebrafish is a central model to dissect the peripheral neuropathy. Genes Genomics 2019; 41: 993–1000.
15. Liao G, Li R, Chen X, et al. Sodium valproate prevents radiation-induced injury in hippocampal neurons via activation of the Nrf2/HO-1 pathway. Neuroscience 2016; 331: 40–51.
16. Lastres-Becker I, Innamorato NG, Jaworski T, et al. Fractalkine activates NRF2/
NFE2L2 and heme oxygenase 1 to restrain tauopathy-induced microgliosis. *Brain* 2014; 137: 78–91.

17. DU S, Yao Q, Tan P, et al. Protective effect of tanshinone IIA against radiation-induced ototoxicity in HEI-OC1 cells. *Oncol Lett* 2013; 6: 901–906.

18. Gupta T and Mullins MC. Dissection of organs from the adult zebrafish. *J Vis Exp* 2010; 37: 1717.

19. Zhang DL, Hu CX, Li DH, et al. Lipid peroxidation and antioxidant responses in zebrafish brain induced by Aphanizomenon flos-aquae DC-1 aphantoxins. *Aquat Toxicol* 2013; 144–145: 250–256.

20. Zhao Z, Liao G, Zhou Q, et al. Sulforaphane attenuates contrast-induced nephropathy in rats via Nrf2/HO-1 pathway. *Oxid Med Cell Longev* 2016; 2016: 9825623.

21. Zhang Y, Gao L, Cheng Z, et al. Kukoamine A prevents radiation-induced neuroinflammation and preserves hippocampal neurogenesis in rats by inhibiting activation of NF-kappaB and AP-1. *Neurotox Res* 2017; 31: 259–268.

22. Tan PX, Du SS, Ren C, et al. MicroRNA-207 enhances radiation-induced apoptosis by directly targeting Akt3 in cochlea hair cells. *Cell Death Dis* 2014; 5: e1433.

23. Guo P, Huang Z, Tao T, et al. Zebrafish as a model for studying the developmental neurotoxicity of propofol. *J Appl Toxicol* 2015; 35: 1511–1519.

24. Balentova S and Adamkov M. Molecular, cellular and functional effects of radiation-induced brain injury: a review. *Int J Mol Sci* 2015; 16: 27796–27815.

25. Lee WH, Sonntag WE, Mitschelen M, et al. Irradiation induces regionally specific alterations in pro-inflammatory environments in rat brain. *Int J Radiat Biol* 2010; 86: 132–144.

26. Wilke C, Grosshans D, Duman J, et al. Radiation-induced cognitive toxicity: pathophysiology and interventions to reduce toxicity in adults. *Neuro Oncol* 2018; 20: 597–607.

27. Mansour HH, Ismael N and Hafez HF. Ameliorative effect of septilin, an ayurvedic preparation against gamma-irradiation-induced oxidative stress and tissue injury in rats. *Indian J Biochem Biophys* 2014; 51: 135–141.

28. Ran Y, Wang R, Gao Q, et al. Dragon’s blood and its extracts attenuate radiation-induced oxidative stress in mice. *J Radiat Res* 2014; 55: 699–706.

29. Talarek S, Listos J, Barreca D, et al. Neuroprotective effects of honokiol: from chemistry to medicine. *Biofactors* 2017; 43: 760–769.

30. Cetin A and Deveci E. Expression of VEGF and GFAP in a rat model of traumatic brain injury treated with honokiol: a biochemical and immunohistochemical study. *Folia Morphol (Warsz)* 2019; 78: 684–694.

31. Chen HH, Chang PC, Chen C, et al. Protective and therapeutic activity of honokiol in reversing motor deficits and neuronal degeneration in the mouse model of Parkinson’s disease. *Pharmacol Rep* 2018; 70: 668–676.

32. Acharya MM, Christie LA, Lan ML, et al. Human neural stem cell transplantation ameliorates radiation-induced cognitive dysfunction. *Cancer Res* 2011; 71: 4834–4845.

33. Amorati R, Zotova J, Baschieri A, et al. Antioxidant activity of magnolol and honokiol: kinetic and mechanistic investigations of their reaction with peroxy radicals. *J Org Chem* 2015; 80: 10651–10659.

34. Pan J, Lee Y, Wang Y, et al. Honokiol targets mitochondria to halt cancer progression and metastasis. *Mol Nutr Food Res* 2016; 60: 1383–1395.

35. Chuang DY, Chan MH, Zong Y, et al. Magnolia polyphenols attenuate oxidative and inflammatory responses in neurons and microglial cells. *J Neuroinflammation* 2013; 10: 15.

36. Sulakhiya K, Kumar P, Jangra A, et al. Honokiol abrogates lipopolysaccharide-induced depressive like behavior by impeding neuroinflammation and oxido-nitrosative stress in mice. *Eur J Pharmacol* 2014; 744: 124–131.

37. Liou KT, Shen YC, Chen CF, et al. Honokiol protects rat brain from focal cerebral ischemia-reperfusion injury by inhibiting neutrophil infiltration and reactive
oxygen species production. Brain Res 2003; 992: 159–166.

38. Reiter RJ, Tan DX, Rosales-Corral S, et al. Melatonin mitigates mitochondrial meltdown: interactions with SIRT3. Int J Mol Sci 2018; 19.

39. Chen ML, Zhu XH, Ran L, et al. Trimethylamine-N-oxide induces vascular inflammation by activating the NLRP3 inflammasome through the SIRT3-SOD2-mtROS signaling pathway. J Am Heart Assoc 2017; 6: e006347.

40. Cao K, Lei X, Liu H, et al. Polydatin alleviated radiation-induced lung injury through activation of Sirt3 and inhibition of epithelial-mesenchymal transition. J Cell Mol Med 2017; 21: 3264–3276.

41. Parodi-Rullan RM, Chapa-Dubocq X, Rullan PJ, et al. High sensitivity of SIRT3 deficient hearts to ischemia-reperfusion is associated with mitochondrial abnormalities. Front Pharmacol 2017; 8: 275.