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

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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.

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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,[13] 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 5×10^3 cells/well in 200 μL complete medium. The cells were treated with or without 50 μM 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.[15] 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 ($n = 40$ 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 methanesulfonate (MS-222), and antibodies against SIRT3 and cyclooxygenase-2 (COX-2) were obtained from Sigma-Aldrich. The antibody against β-actin was purchased from Cell Signaling Technology (Beverly, MA, USA). The kits for evaluating ROS, interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) were obtained from Nanjing Bioengineering Institute (Nanjing, China).

Zebrafish irradiation

Irradiation of zebrafish brains was performed as previously described.[15] 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.[18]

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′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining, 24 hours after irradiation. Staining was performed according to the manufacturer's instructions and as previously described.[19,20]

The TNF-α and IL-1β 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-1β and TNF-α levels were measured using commercial ELISA kits as per the manufacturer's instructions.[21]

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.[22] The primer sequences are shown in Table 1.

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

Gene	Zebrafish	HT22 cells
β-actin	F: GTGCCCATCTACGAGGGTTA	F: AGCCATGTACGTAGCCATCC
	R: TCTCAGCTGTGGTGGTGAAG	R: CTCTCAGCTGTGGTGGTG
SIRT3	F: CATTAAATGTGGTGGAACAAGAGGCCTG	F: ATCCCGGACTTCAGATCCCC
	R: AGTTCCTCTCCTTTGTAATCCCTCCGAC	R: CAACATGAAAAAGGGCTTGGG
COX-2	F: TATGGAGAGACGCTGGAGGTTCA	F: ATCTGGCTTCGGGAGCACAA
	R: CAAATTTCTGCTCTTCCGGGAT	R: GTGGTAACCGCTCAGGTGTT

COX-2, cyclooxygenase-2; F, forward; R, reverse; SIRT3, sirtuin 3.

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.[23] The protein concentrations were detected and western blot assays were performed as previously described.[22] 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

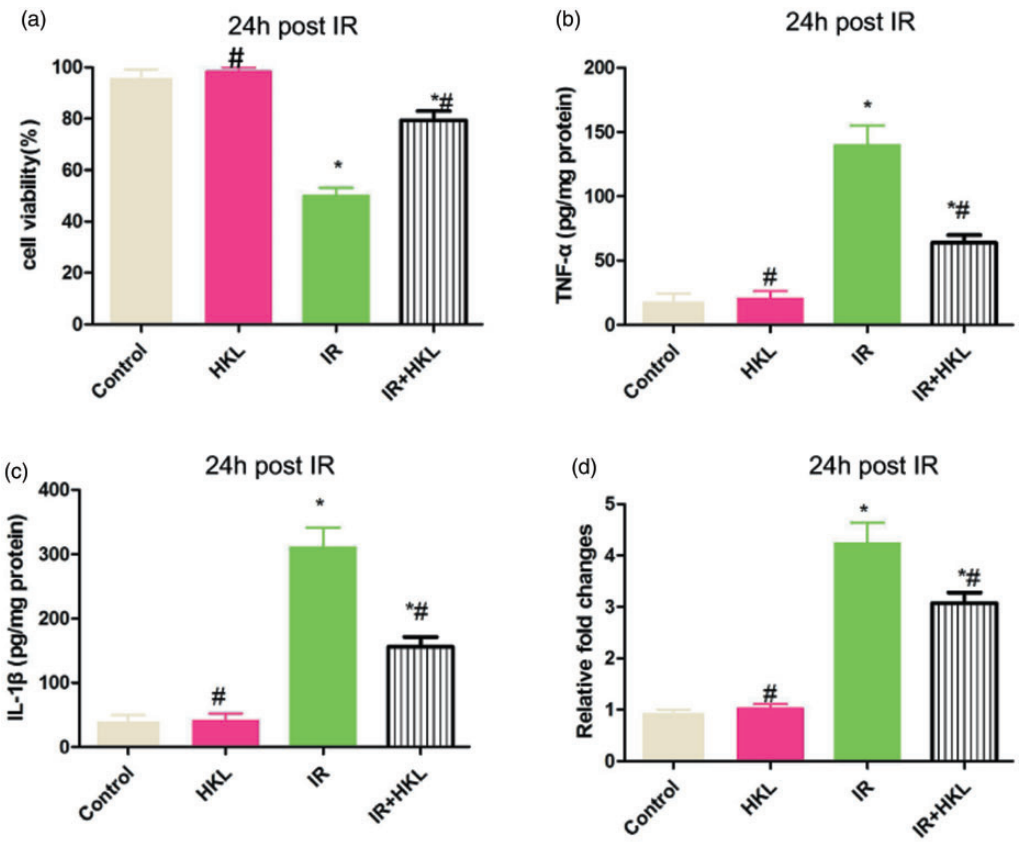

Figure 1. HKL has radioprotective roles in irradiated HT22 cells. (a) HKL increased cell survival; (b) HKL reduced TNF-α; (c) HKL reduced IL-1β; (d) HKL decreased ROS. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the IR group.
HKL, honokiol; IL-1β, interleukin-1 beta; IR, irradiation; TNF-α, tumor necrosis factor-alpha.

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)).

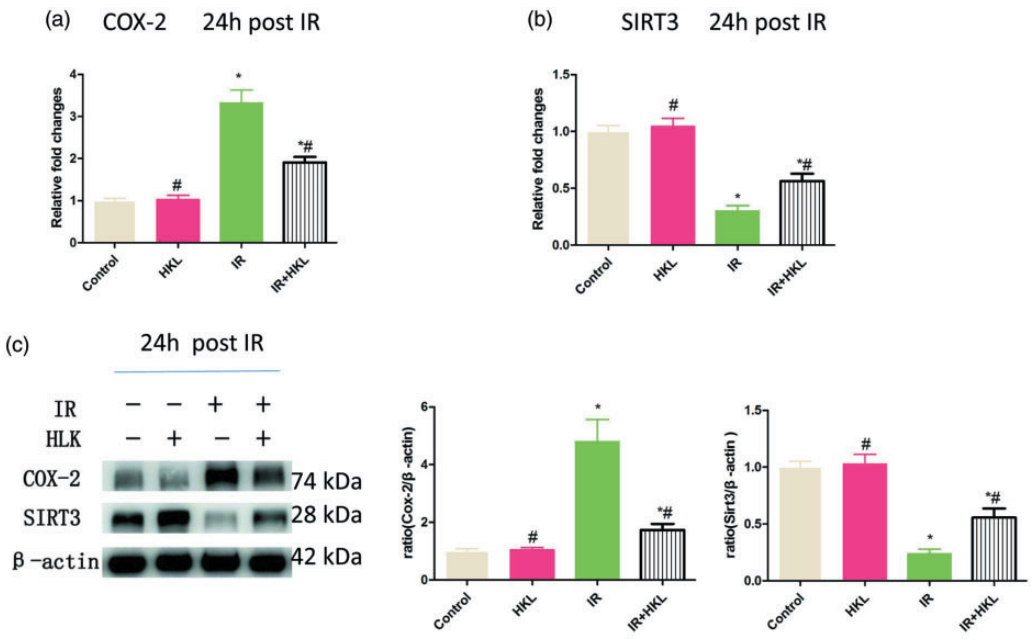

Figure 2. HKL decreased the expression of COX-2 and increased the expression of SIRT3 in HT22 cells. (a) HKL decreased the expression of *COX-2* mRNA; (b) HKL decreased the expression of *SIRT3* mRNA; (c) HKL reduced COX-2 protein levels and increased SIRT3 protein levels. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the IR group.
COX-2, cyclooxygenase-2; HKL, honokiol; IR, irradiation; SIRT3, sirtuin 3.

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.[24] Inflammation and oxidative stress are the major mechanisms involved in radiation-induced brain injury.[15,24] 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-α and IL-1β and upregulate pro-inflammatory pathways.[25,26]

Some antioxidants have radioprotective roles.[27,28] For example, HKL has been demonstrated to have antioxidative activity and neuroprotective effects in several CNS diseases.[15,29–31] In the present study, increased ROS production was observed in the hippocampus of zebrafish after irradiation, which corroborated the results of previous reports.

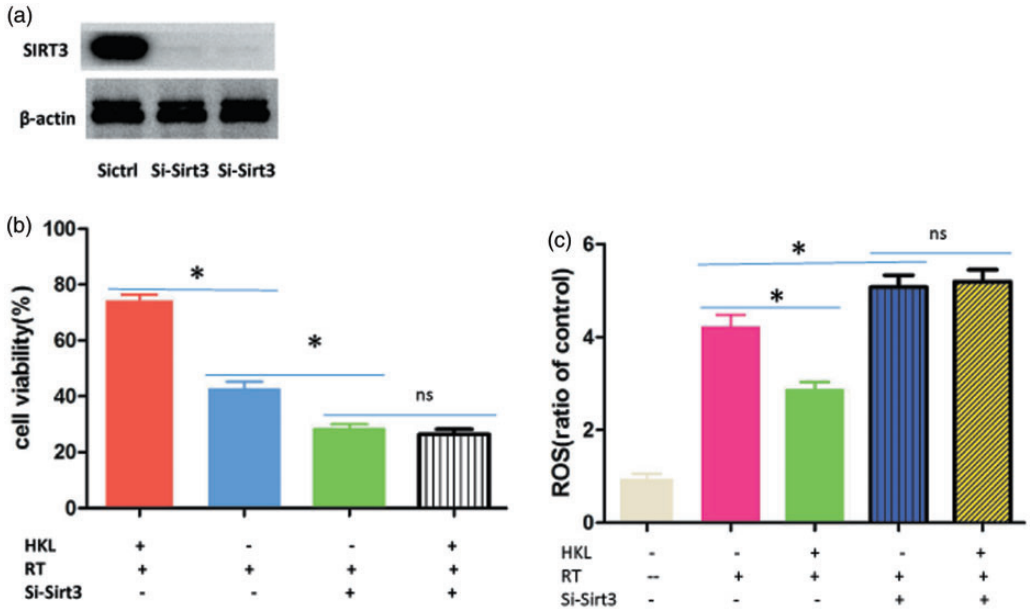

Figure 3. HKL did not have a neuroprotective role in SIRT3-deficient cells. (a) The effects of *SIRT3* siRNA on SIRT3 protein were evaluated by western blot. (b) HKL did not increase cell survival in SIRT3-deficient cells. (c) HKL did not attenuate ROS in SIRT3-deficient cells. $^{*}P < 0.05$.
HKL, honokiol; NS, not significant; ROS, reactive oxygen species; SIRT3, sirtuin 3.

Persistent ROS generation contributes to brain damage and dysfunction.[32]

HKL is a major bioactive constituent of the Chinese medicinal plant *M. officinalis*. In HKL, the hydroxyl group of the second phenol possesses good chemical reactivity with peroxyl radicals.[33] HKL suppresses mitochondrial complex I-dependent respiration, stimulates the formation of mitochondrial ROS, induces 5′ 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.[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.[35–37] Our study also demonstrated that HKL can attenuate not only ROS levels, but also TNF-α and IL-1β levels.

SIRT3 is involved in the antioxidant pathway and is associated with several human diseases.[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.[39] For example, Cao et al. reported that SIRT3

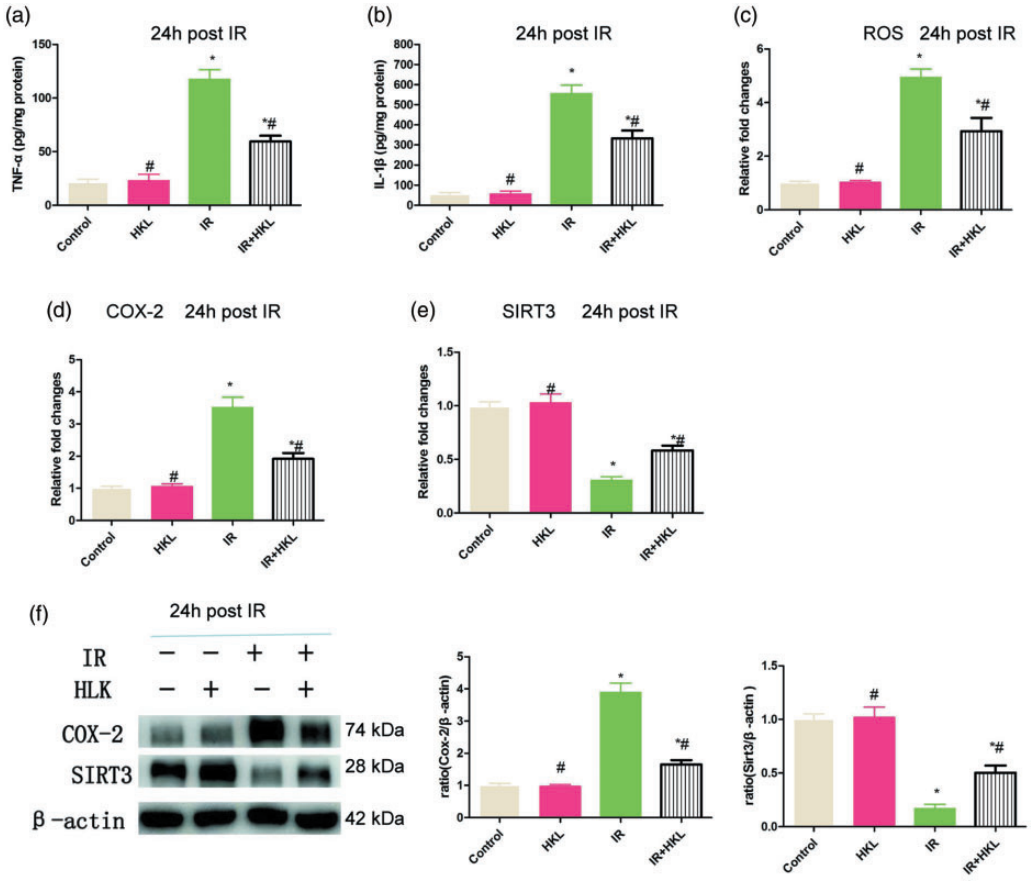

Figure 4. HKL has radioprotective roles in the hippocampus of irradiated zebrafish. (a) HKL reduced TNF-α levels; (b) HKL reduced IL-1β levels; (c) HKL decreased ROS levels; (d) HKL decreased *COX-2* mRNA expression; (e) HKL decreased *SIRT3* mRNA expression; (f) HKL reduced COX-2 protein levels and increased SIRT3 protein levels. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the IR group. HKL, honokiol; IL-1β, interleukin-1 beta; IR, irradiation; ROS, reactive oxygen species; SIRT3, sirtuin 3; TNF-α, tumor necrosis factor-alpha.

activation can alleviate radiation-induced lung injury.[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[11,29]. Additionally, *SIRT3*-knockout mice have exaggerated cardiac dysfunction during ischemia–reperfusion.[41] A SIRT3 activator compound also failed to demonstrate a protective role in *SIRT3*-knockout mice with acute lung injury.[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.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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