Research Article

Pterostilbene Attenuates Subarachnoid Hemorrhage-Induced Brain Injury through the SIRT1-Dependent Nrf2 Signaling Pathway

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Neuroinflammatory injury, oxidative insults, and neuronal apoptosis are major causes of poor outcomes after subarachnoid hemorrhage (SAH). Pterostilbene (PTE), an analog of resveratrol, has been verified as a potent sirtuin 1 (SIRT1) activator. However, the beneficial actions of PTE on SAH-induced brain injury and whether PTE regulates SIRT1 signaling after SAH remain unknown. We first evaluated the dose-response influence of PTE on early brain impairment after SAH. In addition, EX527 was administered to suppress SIRT1 signaling. The results revealed that PTE significantly attenuated microglia activation, oxidative insults, neuronal damage, and early neurological deterioration. Mechanistically, PTE effectively enhanced SIRT1 expression and promoted nuclear factor-erythroid 2-related factor 2 (Nrf2) accumulation in nuclei. Furthermore, EX527 pretreatment distinctly repressed PTE-induced SIRT1 and Nrf2 activation and deteriorated these beneficial outcomes. In all, our study provides the evidence that PTE protects against SAH insults by activating SIRT1-dependent Nrf2 signaling pathway. PTE might be a therapeutic alternative for SAH.

1. Introduction

Subarachnoid hemorrhage (SAH) remains a catastrophic neurovascular disorder [1]. Early brain injury (EBI) is one of the main causes for poor prognosis after SAH. Thus, therapeutic options for targeting EBI might improve long-term neurological outcomes in SAH patients. Mounting evidence indicates that severe oxidative insults, imbalanced inflammatory injury, and cell apoptosis play vital roles in SAH-induced early brain damage [2–4]. However, effective pharmacotherapies for reversing the progression of EBI remain lacking. Thus, identifying new drugs for SAH patients is urgently needed.

Resveratrol (RSV) is extracted from pines and blueberry. It has been demonstrated that RSV is a powerful sirtuin1 (SIRT1) activator and could effectively protect against SAH-induced brain injury by suppressing oxidative damage, neuroinflammation, and neuronal death [5, 6]. Pterostilbene (PTE), an analog of RSV, has also been verified as a potent SIRT1 activator [7]. In addition, PTE has better oral bioavailability and higher bioactivity than RSV. Accumulating evidence has demonstrated that PTE exhibits different protective activities [8–11]. In neurological diseases, PTE serves cerebroprotective effects against Alzheimer's disease, cerebral ischemia, and aging-related diseases. However, the role of PTE after SAH and the precise mechanisms remain unknown. Therefore, we aimed to illuminate whether PTE ameliorated brain damage following SAH and the effects of PTE on SIRT1-mediated signaling.

2. Methods and Materials

2.1. Animal Model. All experiments were implemented by the Animal Ethics Review Committee of Yijishan Hospital (approved number: 2019A0423) [12, 13]. Health male SD rats (250–300 g) were conducted for model construction. We used a prechiasmatic cistern injection model of SAH in male rats [14, 15]. According to previous protocols, rats were firstly anaesthetized with avertin. Then, 0.35 ml arterial blood (extract from the femoral artery) was slowly infused into the prechiasmatic cistern. Sham-treated rats underwent similar procedures, with the injection of physiologic saline. Buprenorphine (0.1 mg/kg) was used to reduce analgesia after model construction.

2.2. Experiment Groups. In this study, 159 rats were used. Nineteen rats died after model construction, and 8 rats were excluded. Animals were randomized into experimental groups. In the first set experiment, rats were randomized into sham group (vehicle), sham + PTE group (10 mg/kg), SAH group, SAH + vehicle group, SAH + PTE group (5 mg/kg), SAH + PTE group (10 mg/kg), and SAH + PTE group (20 mg/kg). In the second set experiment, rats were randomized into sham group (vehicle), SAH group (vehicle), SAH + PTE group (10 mg/kg), and SAH + PTE + EX527 group (10 mg/kg PTE). Animals groups and mortality rates were shown in Supplementary Table 1.

2.3. Drug Treatment. PTE was resolved in dimethylsulfoxide (DMSO) with physiological saline. Three doses of PTE (5, 10, or 20 mg/kg) or vehicle was injected intraperitoneally at 2 h and 12 h after model construction. The doses of PTE were chosen based on previous researches [7, 16]. EX527 (dissolved in 1% DMSO, 15 mg/kg) was injected intraperitoneally for 3 days before model construction [17].

2.4. Neurobehavior Function. The modified Garcia score was employed to assess behavior deficits in different groups [18]. Rotarod performance was conducted as previously reported [19, 20]. The latency to fall from rotarod was recorded. The detailed procedures were conducted according to previous studies [21]. Neurological function was recorded by two investigators blinded to the grouping.

2.5. Biochemical Evaluation. The endogenous antioxidants and the lipid peroxidation biomarker malondialdehyde (MDA) were determined via using different commercial assay kits (Beyotime Biotechnology). The hydrogen peroxide (H_2O_2) expression was detected via using a commercial H_2O_2 assay kit (Abcam).

2.6. Western Blotting. Western blotting was conducted according to previous studies [22]. First, RIPA lysis buffer was used to extract protein samples in brain tissues. Protein concentrations in each sample were measured before western blot analysis. Equal protein extracts in different groups were moved to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk, primary antibodies against SIRT1 (SC-15404), nuclear factor-erythroid 2-related factor 2 (Nrf2, ab31163), heme oxygenase-1 (HO-1, ab13243),

SOD-1 (ab51254), NAD(P)H dehydrogenase quinone 1 (NQO-1, ab34173), Histone H3 (BS1660), and β-actin (AP0060) were employed to incubate with these membranes. Then, bands were hatched with the species-corresponding secondary antibodies. Band intensities were analyzed by ImageJ.

2.7. ELISA Assay. After sample preparation, the expressions of proinflammatory cytokines were determined using the respective commercial kits (Multi Sciences. China). The concrete steps were performed in consistent with the manufacturer's instructions.

2.8. Immunofluorescence Staining. In line with previous studies [23], frozen sections of brain tissue were first fixed in paraformaldehyde (4%). Following incubated with Triton X-100 (0.1%), sections were then blocked with 1% bovine serum albumin. The samples were then hatched with primary antibodies against Iba-1 (1 : 100), Nrf2 (1 : 100), and SIRT1 (1 : 50) overnight followed by proper secondary antibodies. After several time washing with phosphate buffered saline, sections were then stained by DAPI solution. Immunofluorescence quantification was recorded by using ImageJ.

2.9. TUNEL Staining. TUNEL staining was according to the manufacturer's instructions. First, frozen sections of brain tissue were fixed in paraformaldehyde (4%), treated with Triton X-100 (0.1%), and blocked with 1% bovine serum albumin. After that, brain slices were hatched with primary antibodies against NeuN (1 : 200) followed by incubation with TUNEL reaction mixture. The number of TUNEL-positive cells was recorded by ImageJ.

2.10. Statistical Analysis. Shapiro–Wilk's test was applied for data normality. Experimental data were normally distributed and were indicated as mean ± SD. Software GraphPad Prism 8.02 was used for data analysis. Neurological behavior scores were evaluated by two-way ANOVA. One-way ANOVA with Bonferroni post hoc test was employed to determine where those differences occurred. The difference $P < 0.05$ was considered significant.

3. Results

3.1. PTE Dose-Dependently Reduced Oxidative Damage after SAH. Oxidative damage is closely associated with SAH-induced early brain insults. As shown, PTE treatment (10 and 20 mg/kg) distinctly suppressed SAH-induced increase in MDA ($P = 0.002$ and $P < 0.001$, respectively) and reactive oxygen species (ROS) overproduction ($P = 0.034$ and $P = 0.0161$, respectively). The decreased antioxidants including catalase (CAT) ($P = 0.03$ and $P = 0.011$, respectively), superoxide dismutase (SOD) ($P = 0.005$ and $P = 0.0012$, respectively), glutathione (GSH) ($P = 0.009$ and $P = 0.0021$, respectively), and glutathione peroxidase (GSH-px) ($P = 0.0025$ and $P < 0.001$, respectively) were also restored after PTE treatment (10 and 20 mg/kg) (Figures 1(a)–1(f)). However, PTE administration at dose of 5 mg/kg did not statistically ameliorate oxidative insults in the early period after SAH ($P > 0.05$). In addition, no statistical differences in
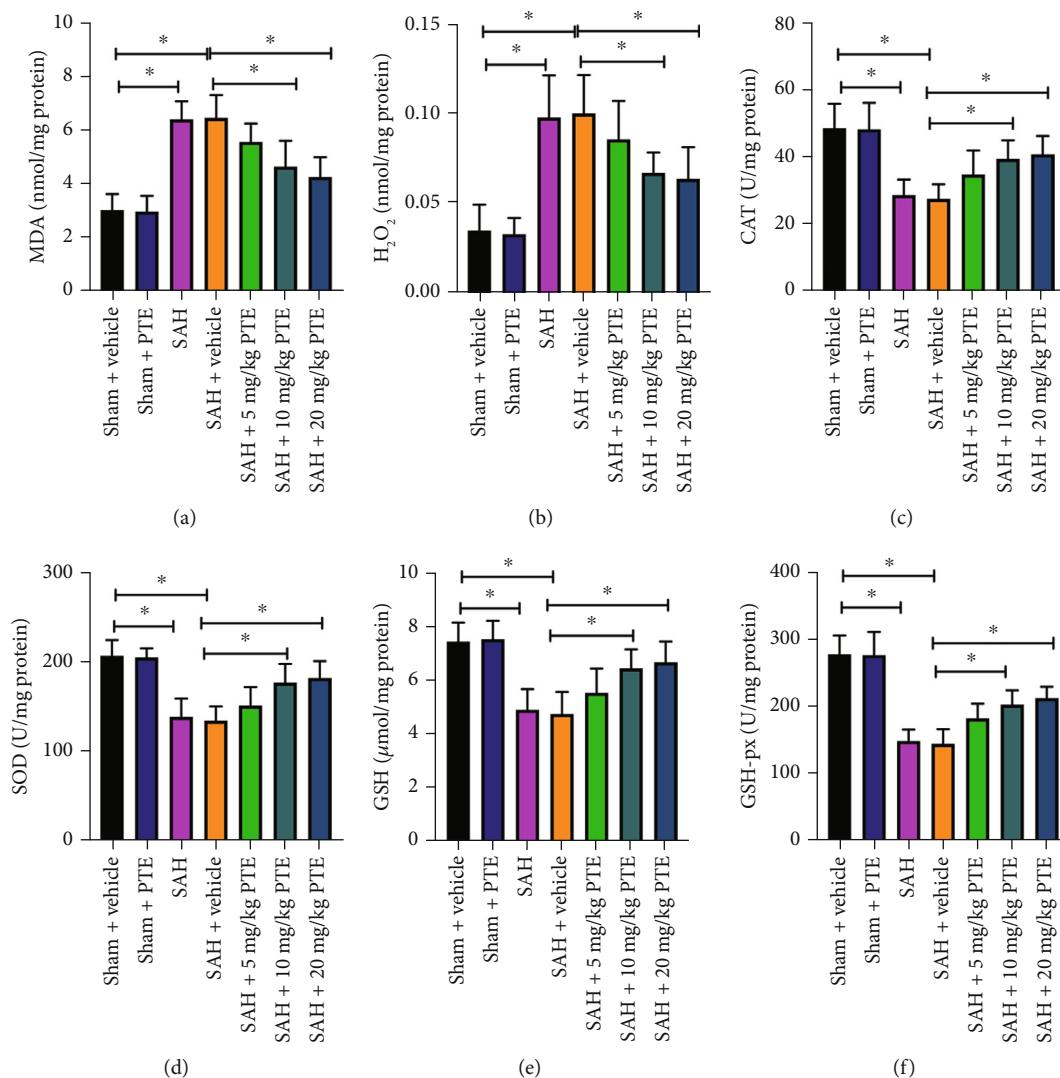
FIGURE 1: Dose-response effects of PTE on SAH-triggered oxidative insults. PTE treatment resulted in reduced MDA (a), and H_2O_2 (b) levels, and better CAT (c), SOD (d), GSH (e), and GSH-Px (f) activities ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Error bars indicated the SD. $^*P < 0.05$.

oxidative insults-related parameters between 10 mg/kg PTE-treated and 20 mg/kg PTE-treated groups were observed. Therefore, a dose of 10 mg/kg PTE treatment was used for the second set of experiment.

3.2. PTE Dose-Dependently Reduced Neuroinflammation after SAH.

The disturbed inflammatory response further aggravates brain damage. In this experiment, it revealed that SAH insults evidently induced a variety of proinflammatory cytokines release, which could be decreased by 10 mg/kg ($P < 0.001$ for IL-1β, $P < 0.001$ for IL-6, $P = 0.0089$ for TNF-α, and $P < 0.001$ for ICAM-1) or 20 mg/kg ($P < 0.001$ for IL-1β, $P < 0.001$ for IL-6, $P < 0.001$ for TNF-α, and $P < 0.001$ for ICAM-1) PTE treatment (Figures 2(a)–2(d)). The brain resident microglia could release proinflammatory mediators and contribute to the disturbed inflammatory injury after SAH. Our data further indicated that PTE

administration dose-dependently inhibited SAH-induced microglia activation, including the Iba1-positive number ($P < 0.001$) and microglia cell body area ($P < 0.001$) (Figures 2(e)–2(g)). These results suggested that PTE could protect against neuroinflammation after SAH.

3.3. PTE Improved Functional Recovery and Reduced Neuronal Apoptosis after SAH.

Inflammatory injury and oxidative insults could exacerbate neuronal death. SAH-induced neuronal death is closely associated with poor neurological deficits. As depicted in Figure 3, SAH aggravated neurological function ($P < 0.05$) and induced a high level of neuronal apoptosis ($P < 0.001$). By contrast, PTE treatment distinctly reduced the number of apoptotic neurons ($P < 0.05$) and improved the short-term neurological functions ($P < 0.05$) (Figures 3(a)–3(d)). These indicated that the cerebroprotective effects of PTE against EBI might
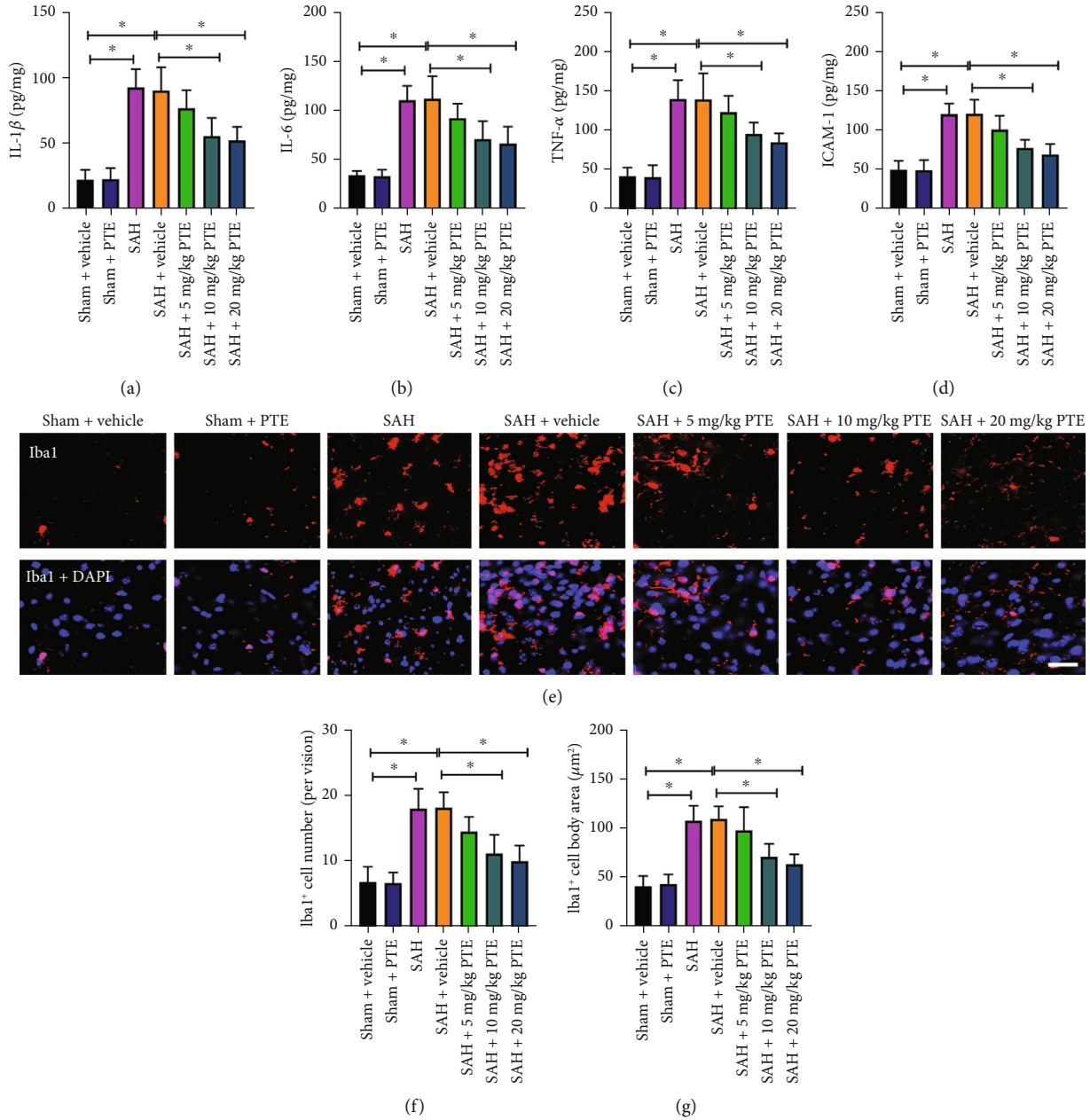
FIGURE 2: Influence of PTE on neuroinflammation following SAH. PTE treatment resulted in reduced IL-1β (a), IL-6 (b), TNF-α (c), and ICAM-1 (d) levels ($n = 6$/groups). Representative Iba1 images (e) and quantitative analyses of the density of Iba1$^+$ cells (f) and Iba$^+$ cell body size (g) in different groups ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Scale bar, 50 μm. Error bars indicated the SD. $^*P < 0.05$.

partially be dependent on reduced cerebral inflammatory injury and oxidative insults.

3.4. PTE Treatment Induced SIRT1 Activation and Nrf2 Signaling Pathway.

PTE, an analog of RSV, has been verified as a potent SIRT1 activator. Meanwhile, accumulating evidence supports a critical role for SIRT1 in modulating Nrf2 activation. It revealed that PTE treatment markedly induced SIRT1 expression ($P < 0.001$) and nuclear distribution of Nrf2 ($P = 0.0036$) in the brain cortex (Figures 4(a)–4(c)). Moreover, PTE evidently enhanced the levels of HO-1, SOD-1, and NQO-1 ($P = 0.03$, $P < 0.01$, and $P < 0.001$, respectively) (Figures 4(d)–4(f)). Furthermore, SIRT1 and Nrf2 staining results indicated that PTE treatment significantly induced SIRT1 activation ($P < 0.001$) and Nrf2 translocation into nuclei ($P < 0.001$) (Figures 4(g)–4(j)). These suggested that PTE could activate SIRT1/Nrf2 signaling. In addition, we used EX527 to suppress SIRT1 activation. As depicted in Figure 4, EX527 prevented the increased SIRT1 expression and Nrf2 signaling pathway by PTE ($P < 0.05$) (Figures 4(a)–4(j)).
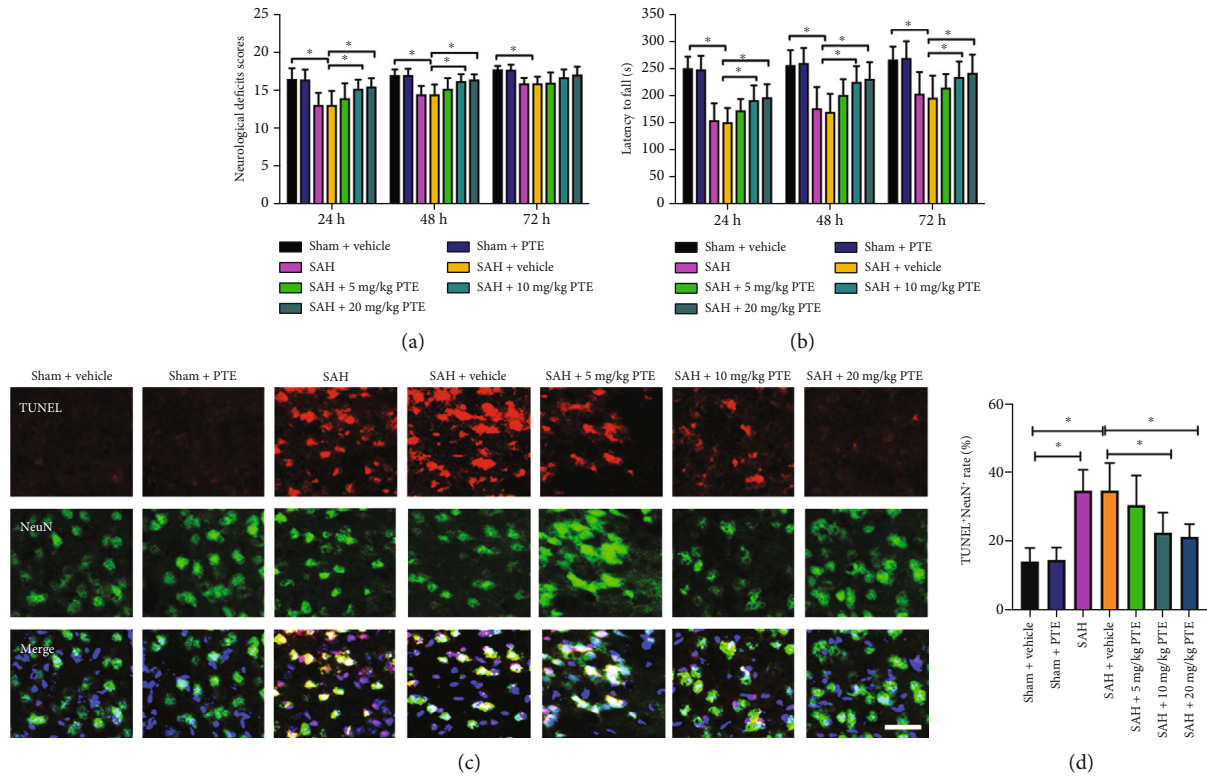
(a)

(b)

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FIGURE 3: Influence of PTE on neurological outcomes and TUNEL apoptosis. PTE reduced deficits scores (a) and improved motor behavior (b) ($n = 8 - 10$/groups). Two-way ANOVA with Bonferroni post hoc test was employed. Representative microphotographs (c) and quantitative analyses (d) of the density of TUNEL^{+}NEUN^{+} cells ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Scale bar, 50 μm. Error bars indicated the SD. $^{*}P < 0.05$.

3.5. Inhibition of SIRT1 Abrogated PTE's Antioxidative Effects.

Whether SIRT1 inhibition prevented PTE's antioxidant effects after SAH remains unknown. We observed that PTE provided a significant antioxidant property against SAH ($P = 0.005$ for MDA, $P = 0.0247$ for H$_2$O$_2$, $P = 0.006$ for CAT, $P = 0.023$ for SOD, $P = 0.0057$ for GSH, and $P < 0.001$ for GSH-px) (Figures 5(a)–5(f)). However, the effects of PTE on oxidative insults and the endogenous antioxidants were abolished by EX527 ($P = 0.02$ for MDA, $P = 0.0239$ for H$_2$O$_2$, $P = 0.014$ for CAT, $P = 0.014$ for SOD, $P = 0.045$ for GSH, and $P = 0.0452$ for GSH-px). EX527 markedly aggravated oxidative insults and further impaired the endogenous antioxidants after SAH (Figures 5(a)–5(f)). These results further indicated that the antioxidant property of PTE might partially be dependent on SIRT1 signaling.

3.6. SIRT1 Inhibition Reversed the Anti-Inflammatory Effects of PTE on SAH.

We further investigated the influence of SIRT1 inhibition on the anti-inflammatory effects of PTE. In congruent, it showed that PTE treatment significantly reduced proinflammatory cytokines release and microglia activation after SAH ($P < 0.001$ for IL-1β, $P < 0.001$ for IL-6, $P = 0.0068$ for TNF-α, and $P < 0.001$ for ICAM1). However, the anti-inflammatory effects by PTE were prevented by EX527 ($P < 0.001$ for IL-1β, $P = 0.0022$ for IL-6, $P = 0.019$ for TNF-α, and $P < 0.001$ for ICAM1) (Figures 6(a)–6(g)). EX527 further increased

inflammatory mediators and activated microglia ($P < 0.001$) after SAH. These results indicated that the anti-inflammatory property of PTE might also partially be dependent on SIRT1 signaling.

3.7. SIRT1 Inhibition Reversed the Cerebroprotection of PTE on SAH.

Oxidative insults, inflammatory injury, and cell death are involved in EBI. Our data indicated that PTE treatment could reduce neuroinflammation, oxidative stress, and cell apoptosis and improve functional recovery after SAH. In addition, suppression of SIRT1 reversed the anti-inflammatory and antioxidative actions of PTE. We further evaluated the effects of SIRT1 inhibition on functional recovery and neuronal survival. Concomitant with the deteriorated oxidative injury and inflammatory impairment, EX527 abrogated the benefits of PTE on functional recovery ($P = 0.0138$ for neurological deficits score, and $P = 0.0072$ for motor function) and neuronal survival ($P = 0.0127$) (Figures 7(a)–7(d)).

4. Discussion

Inflammatory injury, oxidative insults, and cell apoptosis exert vital roles in SAH [2, 3, 21]. The present study revealed that PTE could significantly attenuate inflammatory response, oxidative damage, neuronal death, and early neurological deterioration after SAH. In addition, PTE induced
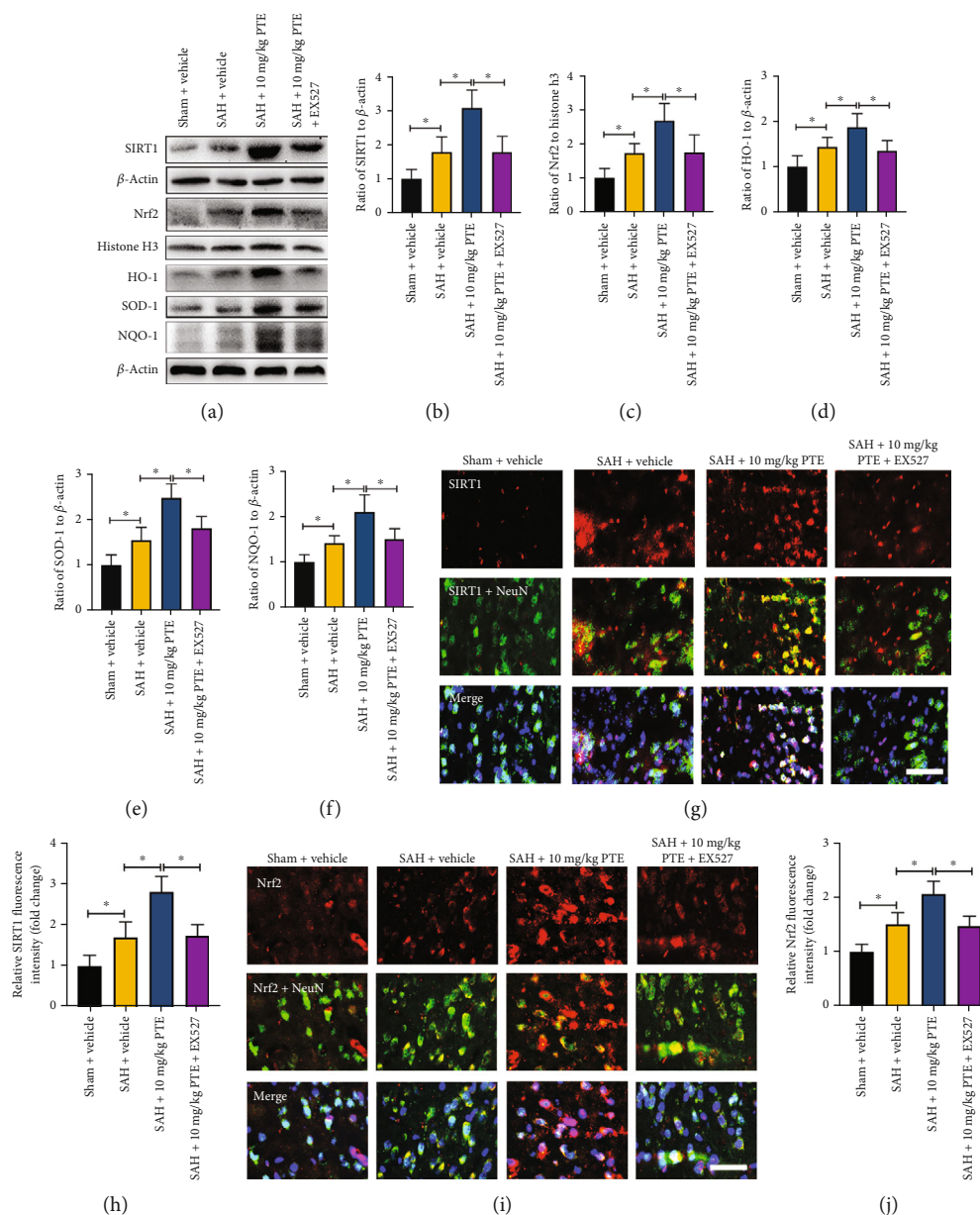
FIGURE 4: Influence of PTE on SIRT1-mediated Nrf2 cascade. Western blot images for SIRT1-mediated Nrf2 cascade (a). PTE markedly induced the expression of SIRT1 (b), nucleus Nrf2 (c), HO-1 (d), SOD-1 (e), and NQO-1 (f). In contrast, EX527 diminished the influence of PTE on SIRT1-mediated Nrf2 cascade ($n = 6$/groups). Immunofluorescence of SIRT1 (g) and Nrf2 staining (i). PTE markedly induced the expressions of SIRT1 (h) and nuclear Nrf2 in neurons (j), which could be abolished by EX527 ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Scale bar, 50 μm. Error bars indicated the SD. $^*P < 0.05$.

SIRT1 activation and Nrf2 signaling pathway. EX527 pretreatment significantly diminished SIRT1-mediated Nrf2 signaling and abolished the neuroprotective actions of PTE following SAH. These data provide evidence that PTE protects against SAH insults by activating SIRT1-dependent Nrf2 signaling pathway.

PTE is a structural analog of RSV with higher bioavailability [16]. RSV is a well-known neuroprotective agent in different CNS diseases. In SAH area, RSV has been shown to ameliorate EBI and cerebral vasospasm and provide multifaceted neurovascular protection by inducing SIRT1-

related signaling pathway [24–26]. In the meantime, RSV could significantly reduce oxidative damage and inflammatory insults after SAH [6, 26]. However, although research has also showed that PTE exhibits comprehensive protective actions, the exact role of PTE in SAH remains obscure. In central nervous system disorders, PTE is able to mitigate a variety of acute brain injuries, including traumatic brain injury (TBI), ischemic stroke, and SAH [16, 27]. Liu et al. reported that PTE reduced microglia-mediated neuroinflammation and neuronal oxidative damage in ischemic stroke [28]. Liu et al. suggested that PTE inhibited NLRP3
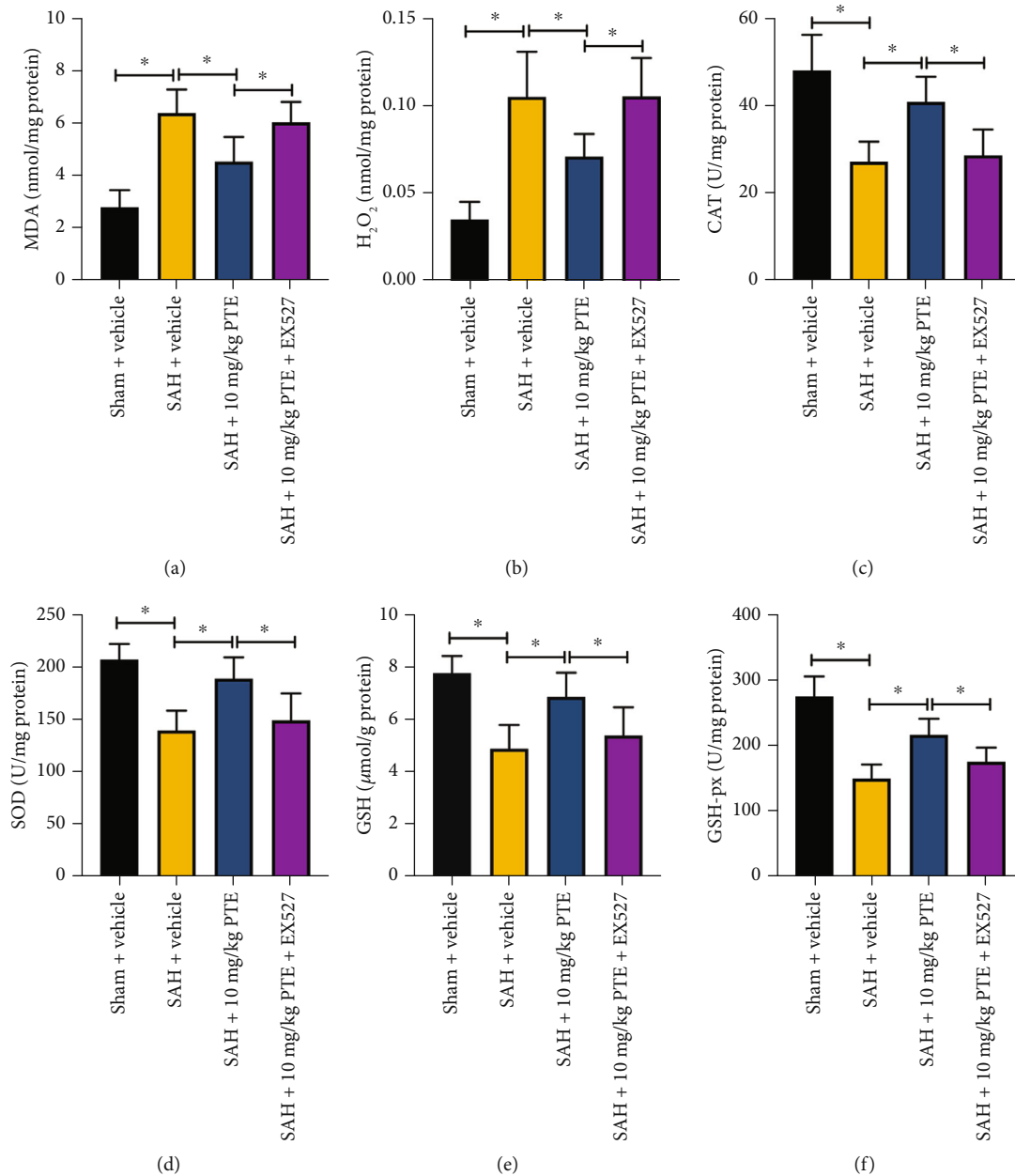
FIGURE 5: SIRT1 inhibition abated the anti-oxidant effects of PTE against SAH. Quantification of MDA (a), H_2O_2 (b), CAT (c), SOD (d), GSH (e), and GSH-Px (f) activities ($n = 6$/groups, one way ANOVA with Bonferroni post hoc test). Error bars indicated the SD. $^*P < 0.05$.

inflammasome and Nox2-mediated oxidative damage after SAH [16]. These previous studies indicated that PTE is a promising drug candidate for acute brain injuries. In congruent, the present study showed that PTE reduced the robust oxidative impairment and improved endogenous oxygen defensive enzymes. Moreover, PTE distinctly inhibited microglia activation and proinflammatory mediators after SAH. Oxidative damage and neuroinflammation directly result in cell apoptosis. Concomitant with the reduced oxidative insults and neuroinflammation, PTE reduced neuronal death and improved functional recovery after SAH.

Although a recent study has showed that PTE inhibited NLRP3 inflammasome and Nox2-signaling after SAH [16], large gaps in knowledge still exist regarding the mechanisms of PTE's cerebroprotective effects. Nrf2 signaling has been implicated in EBI. Under normal circumstances, Nrf2 exerts a vital role in redox homeostasis and immune response. In addition, Nrf2 is fundamentally neuroprotective in other acute brain injuries, including cerebral ischemia, TBI, and intracerebral hemorrhage [15, 29–32]. Wang et al. reported that Nrf2 activation by melatonin receptor attenuated oxidative stress and neuroinflammation in a TBI mouse model [30]. Chen et al. demonstrated that Nrf2 activation by
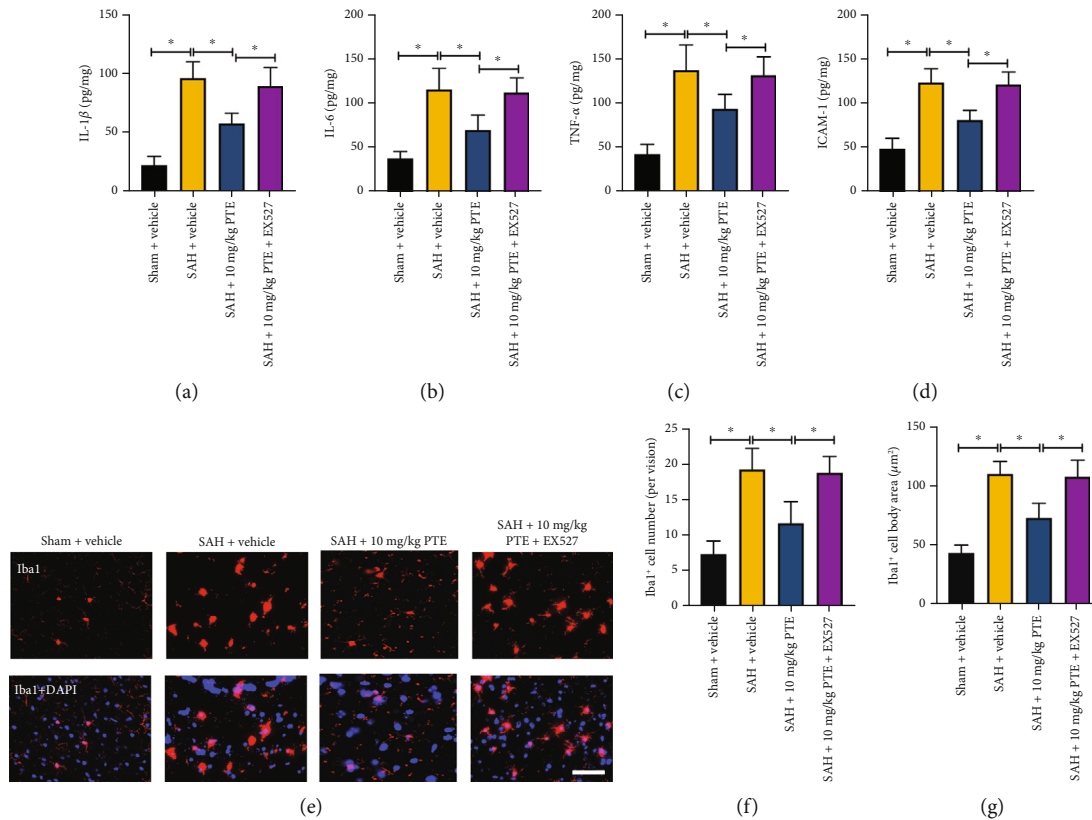
(a)   (b)   (c)   (d)

(e)   (f)   (g)

FIGURE 6: Suppression of SIRT1 abated the anti-inflammatory action of PTE. Quantitative analyses of IL-1β (a), IL-6 (b), TNF-α (c), and ICAM-1 (d) ($n = 6$/groups). Representative microphotographs (e) and quantitative analyses of the density of Iba1$^+$ cells (f) and Iba$^+$ cell body size (g) ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Scale bar, 50 μm. Error bars indicated the SD. $^*P < 0.05$.

omaveloxolone inhibited M1-like microglia and ROS generation in intracerebral hemorrhage models [29]. Therefore, we evaluated changes in the Nrf2 signaling after PTE treatment. Our data revealed that PTE distinctly increased Nrf2 expression in nuclei as well as the subsequent pathways. However, it should be noted that Nrf2 plays a more important role in redox homeostasis. Recently, more and more studies indicated that Nrf2 also participated in modulating immune response by targeting different molecular targets. For example, Zhang et al. reported that Nrf2 activation could mediate HO-1 signaling to mitigate LPS-induced inflammation and lung injury [33]. Xiao et al. showed that Nrf2 could inhibit nod-like receptor pyrin domain-containing 3 inflammasome-mediated neuroinflammation and pyroptosis after cerebral ischemia-reperfusion injury [34]. In addition, Nrf2 has been demonstrated to regulate transforming growth factor-β, toll-like receptor 4/NF-κB, and Notch to affect microglial/macrophage polarization in different diseases models [35, 36]. However, whether PTE modulates these molecular targets after SAH remains obscure. Additional preclinical studies are still needed to clarify this. In summary, these data supported that Nrf2 signaling is involved in the neuroprotective effects of PTE.

SIRT1 was shown to implicate in different cellular biological functions [17, 21, 37, 38]. Evidence supports a

critical role for SIRT1 in modulating Nrf2 activation in different diseases [39–42]. Zhang et al. reported that astaxanthin protected against TBI by modulating SIRT1/Nrf2/p38 signaling pathway [31]. Mao et al. indicated that si-SIRT1 abated SIRT1/Nrf2 activation by fucoxanthin in a model of renal ischemia-reperfusion injury [40]. PTE, a dimethylated analog of RSV, has also been verified as a potent SIRT1 activator in different research fields [43–45]. However, whether PTE mediates SIRT1 signaling in experimental SAH remains unknown. We further used a SIRT1 selective inhibitor EX527 to investigate the interaction between SIRT1 and Nrf2. In this study, EX527 distinctly suppressed SIRT1 and Nrf2 expressions and subsequent molecular targets after SAH. Moreover, we observed that EX527 pretreatment deteriorated the cerebroprotection of PTE against SAH. Overall, these data indicated that the neuroprotection conferred by PTE after SAH was through SIRT1-dependent Nrf2 signaling.

Our study has several limitations. First, whether PTE administration could improve cognitive function and reduce the delayed cerebral ischemia after SAH remain unknown. Second, we did not evaluate the possible beneficial effects of PTE on female rats. Third, Nrf2 was shown to positively mediate SIRT1 signaling in other diseases [46]. The exact interaction between SIRT1 and Nrf2 signaling in experimental SAH still needs to be clearly elucidated.
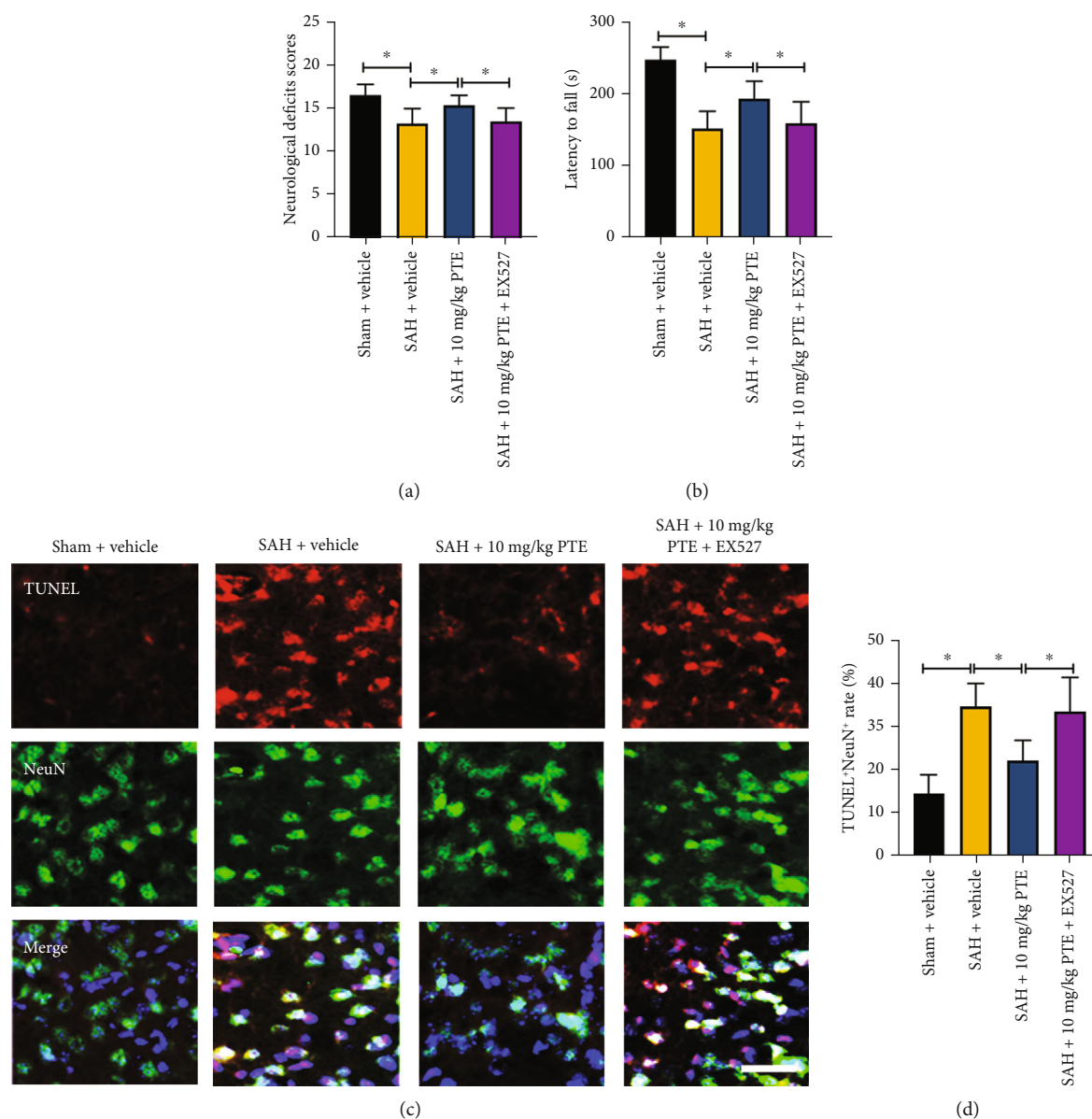
(a)

(b)

(c)

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Figure 7: SIRT1 inhibition abated the beneficial actions of PTE on neurological scores and cellular apoptosis. Quantitative analyses of deficits scores (a) and rotarod function (b) ($n = 10 - 12$/groups). Representative microphotographs (c) and quantitative analyses (d) of TUNEL$^+$NEUN$^+$ cells in different groups ($n = 6$/groups). One-way ANOVA with Bonferroni post hoc test was employed. Scale bar, 50 μm. Error bars indicated the SD. $^*P < 0.05$.

5. Conclusion

In conclusion, we revealed that PTE protected against brain damage after SAH via reducing neuroinflammatory injury, oxidative insults, and cellular apoptosis. By interacting with SIRT1/Nrf2 signaling, PTE attenuated EBI and improved neurological outcomes after SAH.

Data Availability

The data can be directed to the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interests.

Authors' Contributions

ZHZ and JCF conceived and designed the study. JCF, ZHZ, JWZ, XTZ, and GZ performed the experiments. ZHZ, JCF, YL, and ZZ performed statistical analysis. ZHZ, JCF, ZBL, YL, and ZZ reviewed and revised the manuscript. Zihuan Zhang and Jincheng Fang contributed equally to this work.

Acknowledgments

Supplementary Materials

Supplementary Table 1: animals groups and mortality rates. (Supplementary Materials)

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