Eriocitrin alleviates sevoflurane-induced cytotoxicity in HT22 cells via Nrf2 pathway

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Abstract

Purpose: To investigate the effect of eriocitrin on sevoflurane-induced neurotoxicity in mice.

Methods: Mouse hippocampal neurons (HT22) were exposed to different concentrations of sevoflurane for 6 h and then incubated with different concentrations of eriocitrin for another 24 h. Cell viability was determined by CCK8 assay, while fluorescence intensity of dichlorodihydrofluorescein was used to evaluate reactive oxygen species. Enzyme linked immunosorbent assay (ELISA) was used to determine oxidative stress, and cellular apoptosis was determined by flow cytometry.

Results: Sevoflurane exposure decreased HT22 cell viability, whereas incubation with eriocitrin increased viability of sevoflurane-treated HT22 cells (p < 0.05). Sevoflurane-induced increase in dichlorodihydrofluorescein fluorescence intensity was reduced by eriocitrin, but eriocitrin attenuated sevoflurane-induced increase in malondialdehyde, superoxide dismutase, and glutathione peroxidase in HT22 cells. Cell apoptosis increased after sevoflurane exposure, and eriocitrin suppressed apoptosis in sevoflurane-treated HT22 cells through downregulation of cleaved caspase-3 and cleaved caspase-9 (p < 0.05). Eriocitrin incubation enhanced protein expression of nuclear factor E2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NAD(P)H quinone dehydrogenase 1 (NQO1) in sevoflurane-treated HT22 cells (p < 0.05).

Conclusion: Eriocitrin ameliorates sevoflurane-induced oxidative stress and inflammatory response in HT22 cells via activation of Nrf2/HO-1/NQO1 signaling. Thus, agent may be useful in the treatment of sevoflurane-induced toxicity, but in vivo studies are required to buttress this.

Keywords: Eriocitrin, Sevoflurane, Oxidative stress, Inflammatory response, Nrf2/HO-1/NQO1, Neurotoxicity

INTRODUCTION

Sevoflurane is an inhalation anesthetic widely used in clinical practice [1,2]. Sevoflurane has been shown to protect HT22 cells from oxygen-glucose deprivation/reoxygenation-induced cell apoptosis and oxidative stress [3]. However, numerous reports have shown that prolonged exposure to sevoflurane induces apoptosis in brain cells, resulting in neurotoxicity [4,5]. An in vivo study showed that sevoflurane exposure lead to decreased learning ability and memory problems [6]. Therefore, there is an urgent need to investigate the mechanism of sevoflurane-induced cytotoxicity in HT22 cells.
induced neurotoxicity in order to ensure safe clinical usage of sevoflurane.

Nuclear factor E2-related factor 2 (Nrf2), activated in response to oxidative stress, is a transcription factor that regulates antioxidant genes, thus participating in maintenance of oxidative metabolism [7]. Nrf2 also regulates mitochondrial function and biogenesis, exerts an anti-inflammatory effect, and is involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease [8]. Sevoflurane-induced cell apoptosis and neuroinflammation were repressed by activation of Nrf2 signaling [9]. Therefore, Nrf2 is a possible therapeutic target of anesthetic-induced neurotoxicity.

Lemon (Citrus limon) has been widely used as an important source of natural flavonoids, and it has antiparasitic, anticancer, antimicrobial, and anti-inflammatory effects [10]. Eriocitrin is the main flavonoid in lemon, and experimental research showed that eriocitrin reduced oxidative and degenerative damage in osteoarthritis [11]. Eriocitrin also attenuated hepatic steatosis [12] and exerted an antioxidant effect in diabetes [13]. Dextran sulfate sodium-induced inflammation in mice with colitis was repressed by eriocitrin [14], and eriocitrin attenuated glucose- and lipid-induced metabolic impairment in obesogenic mice [15]. Eriocitrin promoted activation of Nrf2/NAD(P)H quinone dehydrogenase 1 (NQO1) /heme oxygenase-1 (HO-1) signaling to reduce ischemia/reperfusion-induced oxidative stress and inflammatory response in the brains of rats [16]. Eriocitrin might also activate Nrf2 pathway to attenuate sevoflurane-induced neurotoxicity.

In this study, HT22 mouse hippocampal neurons were exposed to sevoflurane and then incubated with eriocitrin. The effects of eriocitrin on cell viability, apoptosis, and oxidative stress in HT22 cells were investigated. The mechanism underlying the neuroprotective effect of eriocitrin was also investigated.

**CELL viabiLITY AND apoptosis assays**

Sevoflurane- and eriocitrin-treated HT22 cells were seeded on plates and cultured for 48 h, followed by incubation with CCK8 solution (Beyotime, Beijing, China) for 2 h. Absorbance at 450 nm was measured with a microplate reader (Sigma-Aldrich). Cells were harvested with trypsin and then resuspended in binding buffer from the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Cells were labeled with PI and annexin V-FITC and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

**measurement of oxidative stress**

HT22 cells were incubated with 0.5 μM dichlorofluorescein diacetate (Sigma-Aldrich) for 20 minutes. The fluorescence intensity of dichlorodihydrofluorescein was measured with a FACSCalibur flow cytometer. Levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were determined using enzyme linked immunosorbent assay kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).

**Western blotting**

Protein samples were isolated from HT22 cells using RIPA lysis buffer (Beyotime). Samples were separated with 10 % SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was blocked and probed with specific antibodies: anti-cleaved caspase-3 and anti-cleaved caspase-9 (1 : 2000, Abcam, Cambridge, UK), anti-Nrf2 and anti-HO-1 (1 : 3000, Abcam), and anti-NQO1 and anti-GAPDH (1 : 4000, Abcam). Following incubation with horseradish peroxidase-conjugated secondary antibody (1 : 5000, Abcam) and tetramethylbenzidine, the protein bands were visualized using chemiluminescence (Sigma-Aldrich).

**Statistical analysis**

All data from at least triplicate samples are expressed as mean ± standard error of the mean and analyzed by Student’s t-test or one-way
analysis of variance using SPSS software (Chicago, IL, USA). A \(p\) value of < 0.05 was considered statistically significant.

**RESULTS**

Eriocitrin increased viability of sevoflurane-treated HT22 cells

To investigate cytotoxicity of sevoflurane in HT22 cells, HT22 viability was determined post-sevoflurane exposure. Sevoflurane exposure reduced cell viability in a dose-dependent manner (Figure 1 A). Sevoflurane-treated HT22 cells were then incubated with eriocitrin. Eriocitrin incubation increased viability of sevoflurane-treated HT22 cells, also in a dose-dependent manner (Figure 1 B), demonstrating that eriocitrin protected against sevoflurane-induced cytotoxicity in HT22 cells.

![Figure 1](image)

**Figure 1:** Eriocitrin increased viability of sevoflurane-treated HT22 cells. (A) Sevoflurane (Sev) exposure reduced viability of HT22 cells in a dose-dependent manner. (B) Eriocitrin incubation increased viability of sevoflurane-treated HT22 cells in a dose-dependent manner. **, *** vs. control, \(p < 0.01\), \(p < 0.001\). @@ vs. sevoflurane, \(p < 0.01\)

Eriocitrin reduced oxidative stress of sevoflurane-treated HT22 cells

Fluorescence intensity of dichlorodihydrofluorescein in HT22 cells was increased post-sevoflurane exposure (Figure 2 A). However, eriocitrin incubation reduced the fluorescence intensity in a dose-dependent manner (Figure 2 A). Moreover, upregulation of MDA (Figure 2 B) and downregulation of SOD (Figure 2 C) and GSH-Px (Figure 2 D) in sevoflurane-treated HT22 cells were reversed by eriocitrin incubation, suggesting an antioxidant effect of eriocitrin on sevoflurane-treated HT22 cells.

![Figure 2](image)

**Figure 2:** Eriocitrin reduced oxidative stress of sevoflurane-treated HT22 cells. (A) Eriocitrin incubation reduced the fluorescence intensity of dichlorodihydrofluorescein in sevoflurane-treated HT22 cells in a dose-dependent manner. (B) Eriocitrin incubation reduced MDA levels in sevoflurane-treated HT22 cells in a dose-dependent manner. (C) Eriocitrin incubation enhanced SOD levels in sevoflurane-treated HT22 cells in a dose-dependent manner. (D) Eriocitrin incubation enhanced GSH-Px levels in sevoflurane-treated HT22 cells in a dosage dependent manner. ***, *** vs. control, \(p < 0.01\), \(p < 0.001\). @@, @@ vs. sevoflurane, \(p < 0.01\), \(p < 0.001\)

Eriocitrin suppressed cell apoptosis of sevoflurane-treated HT22 cells

HT22 cell apoptosis was promoted by sevoflurane exposure (Figure 3), whereas incubation with eriocitrin suppressed apoptosis in sevoflurane-treated HT22 cells (Figure 3). Moreover, eriocitrin incubation attenuated sevoflurane-induced upregulation of cleaved caspase-3 and cleaved caspase-9 in sevoflurane-treated HT22 cells (Figure 4), revealing the anti-apoptotic effect of eriocitrin on sevoflurane-treated HT22 cells.

![Figure 3](image)

**Figure 3:** Eriocitrin suppressed cell apoptosis of sevoflurane-treated HT22 cells. Eriocitrin incubation suppressed apoptosis in sevoflurane-treated HT22 cells in a dose-dependent manner
Figure 4: Effect of eriocitrin on expression of apoptotic biomarkers. Eriocitrin incubation reduced protein expression of cleaved caspase-3 and cleaved caspase-9 in sevoflurane-treated HT22 cells in a dose-dependent manner. *** vs. control $p < 0.001$. @, @@ vs. sevoflurane, $p < 0.05$, $p < 0.001$

Eriocitrin promoted activation of Nrf2 signaling in sevoflurane-treated HT22 cells

Sevoflurane exposure reduced protein expression of Nrf2 in HT22 cells (Figure 5), and the downstream genes Nrf2, HO-1, and NQO1 were also downregulated in sevoflurane-treated HT22 cells (Figure 5). However, eriocitrin incubation upregulated Nrf2, HO-1, and NQO1 expression in sevoflurane-treated HT22 cells (Figure 5), indicating that eriocitrin protected cells against sevoflurane-induced cytotoxicity through activation of Nrf2 signaling.

Figure 5: Eriocitrin promoted activation of Nrf2 signaling in sevoflurane-treated HT22 cells. Eriocitrin incubation enhanced protein expression of Nrf2, HO-1, and NQO1 in sevoflurane-treated HT22 cells in a dose-dependent manner. **, ***$p < 0.01$, $p < 0.001$, compared with control; @@, @@@$p < 0.01$, $p < 0.001$, compared with sevoflurane

DISCUSSION

Limio leaf extract produced a neuroprotective effect against neuroblastoma cell lines [16]. Flavonoids protected against nitroprusside-induced neurotoxicity [17] and ameliorated sevoflurane-induced memory dysfunction. Eriocitrin, the main flavonoid in lemon, exerted antioxidant and anti-inflammatory effects in ischemia/reperfusion-induced brain injury [16]. Therefore, the role of eriocitrin in sevoflurane-induced neurotoxicity was investigated.

A previous study showed that neuronal apoptosis was associated with the neurotoxic effect of sevoflurane on the brain [18]. Sevoflurane promoted endoplasmic reticulum stress in HT22 cells and induced hippocampal neuronal apoptosis [19]. Inhibition of neuronal apoptosis ameliorated sevoflurane-induced neurotoxicity and neurocognitive impairment [18]. Viability of HT22 cells was reduced, whereas apoptosis was promoted by sevoflurane exposure. The effect of eriocitrin on neuronal apoptosis has been reported in rat post-middle cerebral artery occlusion [16]. Eriocitrin increased viability of sevoflurane-treated HT22 cells and suppressed apoptosis through downregulation of cleaved caspase-3 and cleaved caspase-9.

Sevoflurane induced DNA damage in neuronal cells through upregulation of reactive oxygen species [21]. Inhibition of reactive oxygen species accumulation through antioxidant agents alleviated sevoflurane-induced DNA damage [21]. Eriocitrin exerted an antioxidant effect on neuronal cells in a rat model of cerebral ischemia/reperfusion [16]. The results of this study showed that eriocitrin attenuated sevoflurane-induced increases in reactive oxygen species and MDA and decreases in SOD and GSH-Px in HT22 cells, demonstrating an antioxidant effect against sevoflurane-induced neurotoxicity. Moreover, neuroinflammation was shown to be associated with sevoflurane-induced cognitive impairment [22]. The inflammatory response of neurons in the rat model of cerebral ischemia/reperfusion was repressed by eriocitrin [16]. Therefore, eriocitrin might repress sevoflurane-induced inflammation.

Nuclear translocation of Nrf2 induced by stimuli promotes binding to antioxidant response elements of target genes, such as the antioxidant enzymes HO-1 and NQO1, thereby producing a protective response against neurodegeneration [23]. Upregulation of Nrf2 conferred neuroprotection of euxanthone in sevoflurane-induced neurotoxicity [9]. Eriocitrin suppressed ischemia/reperfusion-induced inflammation and oxidative stress through upregulation of Nrf2 [16, 24]. Protein expression of Nrf2, HO-1, and NQO1 was upregulated in sevoflurane-treated HT22 cells after eriocitrin treatment, suggesting that eriocitrin-mediated activation of Nrf2 signaling was associated with the suppressive effect on sevoflurane-induced neurotoxicity.
CONCLUSION

This study confirmed that eriocitrin exerts anti-apoptotic and antioxidant effects on sevoflurane-treated HT22 cells and protects against sevoflurane-induced neurotoxicity through activation of Nrf2/HO-1/NQO1 signaling. However, the effects of eriocitrin on sevoflurane-induced neuronal apoptosis, oxidative stress, inflammation, and cognitive impairment of rats needs further investigation in vivo.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhiguo Yuan and Mei Zhu designed the study and supervised data collection. Xiaojia Wei analyzed and interpreted the data. Xiaojing Li prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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