Tropical Journal of Pharmaceutical Research August 2020; 19 (8): 1611-1617
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i8.7

Original Research Article

Eriodictyol attenuates spinal cord injury by activating Nrf2/HO-1 pathway and inhibiting NF-κB pathway

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Sent for review: 8 May 2020 *Revised accepted: 28 July 2020*

Abstract

Purpose: *To investigate the effect of eriodictyol on spinal cord injury (SCI) and its underlying mechanism of action.*
Methods: *Thirty Sprague-Dawley rats were assigned to sham, SCI, and eriodictyol-treated groups (SCI + Eri; 10, 20, and 50 mg/kg). Moderate spinal cord contusion injury was induced to model SCI. Locomotor recovery was assessed based on Basso, Beattie, and Bresnahan (BBB) score. Pain was evaluated by paw withdrawal threshold (PWT) and latency (PWL), and spinal cord water content was measured. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) expression were determined by enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Immunoassay was used to determine malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-PX) levels while Western blotting was employed to evaluate nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), nuclear factor-kappa B (NF-κB), and phosphorylated NF-κB (p-NF-κB) levels.*
Results: *Eriodictyol elevated BBB score, PWT, and PWL in SCI rats but reduced spinal cord water content ($p < 0.05$). Eriodictyol treatment down-regulated TNF-α, IL-1β, IL-6, and MDA, whereas SOD, GSH, and GSH-PX levels were elevated ($p < 0.05$). Eriodictyol administration increased Nrf2 and HO-1 levels but reduced p-NF-κB/NF-κB.*
Conclusion: *This study provides a potential therapy to promote long-term functional recovery following SCI.*

Keywords: *Spinal cord injury, Eriodictyol, Nrf2/HO-1 pathway, NF-κB signaling pathway, Polymerase chain reaction, Basso, Beattie and Bresnahan score*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Car accidents and falls frequently cause spinal cord injury (SCI), a devastating and common condition of acute trauma [1]. This trauma can impair bowel and bladder function, mobility, and autonomic nerve function and is often accompanied by pressure ulcers and pain that directly damages patient health [2]. In terms of pathophysiology, secondary injuries that may occur after SCI include edema, secondary ischemia, oxidative stress injury, inflammatory

cell infiltration, and neuronal apoptosis [3-5]. Though spinal surgery and methylprednisolone are key interventions for SCI [4], there are currently no effective strategies for neurologic or functional recovery following SCI. Therefore, it is critical to develop new therapeutic strategies to promote functional recovery in patients with SCI.

Nuclear factor-kappa B (NF-κB), heme oxygenase-1 (HO-1), nuclear factor erythroid 2-related factor 2 (Nrf2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) play pivotal roles in oxidative stress and neuro-inflammation [6]. Though NF-κB and HO-1/Nrf2 signaling have been shown to contribute to inflammatory and antioxidant responses after lipopolysaccharide (LPS) challenge [7], the roles of NF-κB and HO-1/Nrf2 in SCI have not been clearly elucidated.

Eriodictyol, a compound isolated from the plant *Dracocephalum rupestre*, is widely distributed in common foods [8]. Published studies have demonstrated that eriodictyol has a variety of biological effects, including the suppression of oxidative stress, inflammation, cell apoptosis, and osteoclast-related diseases [9]. Eriodictyol has been shown to elevate HO-1 levels by activating the Nrf2/antioxidant response element (ARE) pathway and to protect against hydrogen peroxide-induced neurotoxicity [10]. In LPS-induced neuro-inflammation, eriodictyol blocked downstream translocation of NF-κB and thus attenuated amyloidogenesis and memory impairment [11]. However, the effect of eriodictyol on SCI and its molecular mechanism are still poorly understood.

The present study demonstrates the protective properties of eriodictyol against SCI in rats and investigates the mechanism underlying its protective effects. This study identifies eriodictyol as a potential therapeutic strategy for patients with SCI.

EXPERIMENTAL

Animals

A total of 30 male Sprague-Dawley rats (8 - 10 weeks old; 250 ± 20 g) were bought from the animal breeding center of Fujian Medical University. The rats were maintained at 25 ± 2 °C for 3 days with food and water *ad libitum* before experiments. All experimental procedures conformed to the guidelines of National Institutes of Health Guide for The Care and Use of Laboratory Animals [12], and the study was approved by the Ethics Committee of The First People's Hospital of Wenling (approval no. 20170046).

Establishment of SCI model and treatment

Rats were randomly assigned to sham operation (sham), SCI, and eriodictyol-treated (SCI + Eri, 10, 20, and 50 mg/kg) groups. The SCI model was established using a spinal cord contusion injury [13]. Animals were anesthetized by intraperitoneal administration of 50 mg/kg sodium pentobarbital (Sigma, St. Louis, MO, USA). Moderate contusion injury was induced with a laminectomy of the eighth thoracic vertebra (T8). The T8 spinous process and laminae were excised to expose circular dura with a diameter of 2.4 mm, and a 2 g weight was dropped from a height of 5 cm onto the exposed dura. After injury, the overlying muscles and skin were sewn closed. Laminectomy without compression was performed in the sham operation group.

Rats were administered 10, 20, or 50 mg/kg of eriodictyol (Seebio Biotech Co. Ltd., Shanghai, China) daily for 4 weeks. The sham group was treated with saline. On day 28, the rats were anesthetized and sacrificed.

Evaluation of locomotor function recovery and behavioral pain tests

Basso, Beattie, and Bresnahan (BBB) scores were assigned as a measure of locomotor recovery. The locomotor rating scale was 0 - 21, where a score of “0” indicated no visible hind limb movement and a score of “21” indicated normal movement.

Paw withdrawal threshold (PWT) was measured to assess mechanical allodynia. Paw withdrawal latency (PWL) was determined using the Hargreaves method of responding to radiant heat. The PWT and PWL assessments were performed as previously reported [14].

Assessment of spinal cord water content

At 72 h post-injury, the spinal cord was obtained from the epicenter for water content assessment. The wet weight of the spinal cord sample was measured, and dry weight was measured after 48 h of drying at 80°C. Percentage of spinal cord water content was calculated by the following equation: (wet weight - dry weight) / wet weight × 100%.

Determination of cytokine expression

Peripheral blood was obtained after eriodictyol treatment and subjected to centrifugation at

10,000 × g for 10 min at 4°C to obtain cell-free supernatants. The levels of TNF-α, IL-1β, and IL-6 were determined using ELISA kits (Dakewe Biotech, Shenzhen, China) according to the manufacturer's instructions.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol reagent (Gibco BRL, Grand Island, NY, USA). PCR was conducted on an ABI 7500 Real-Time PCR System. The RT-qPCR primer pairs are shown in Table 1**.** The PCR was performed as follows: 95 °C for 10 min, 90 °C for 15 s and 60 °C for 60 s for 40 cycles, 95 °C for 60 s.

Determination of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-PX) levels

Peripheral blood was obtained after eriodictyol treatment and subjected to centrifugation at 10,000 × g for 10 min at 4 °C. The levels of MDA, SOD, GSH, and GSH-PX were determined using immunoassay kits (Dakewe Biotech).

Western blot analysis

Spinal cord samples (15 mg) were homogenized, and proteins were extracted on ice with lysis buffer (Thermo Scientific, Rockford, IL, USA). Cells were washed twice with cold PBS. After sonication and centrifugation, protein concentration in the supernatant was determined using the Bicinchoninic Acid Protein Assay Kit (Thermo). Equal amounts of total protein were separated by 10 % SDS-PAGE, and the separated proteins were transferred onto polyvinylidene difluoride membranes. Non-specific binding sites were blocked in NaCl/Tris-T buffer containing 5 % non-fat milk for 1 h.

Membranes were incubated overnight at 4 °C with primary antibodies, including monoclonal mouse anti-human antibodies against Nrf2, HO-1, NF-κB, and phosphorylated NF-κB (p-NF-κB) (BD Biosciences Franklin Lakes, NJ, USA). The membranes were then incubated for 1 h at room temperature with anti-mouse IgG conjugated with horseradish peroxidase (BD Biosciences). Protein expression levels were determined using an enhanced chemiluminescence detection system (GE Healthcare, USA).

Statistical analysis

SPSS 17.0 (SPSS Inc, Chicago, IL, USA) was used for all statistical analyses. The experimental data were expressed as means ± SD. Comparisons among multiple groups were performed using ANOVA followed by Tukey's multiple comparison test. The cutoff for statistical significance was $p < 0.05$.

RESULTS

Eriodictyol alleviated locomotor dysfunction in SCI rats

Different concentrations of eriodictyol were administered after SCI induction in rats. Locomotor function recovery was evaluated using BBB scores. The SCI group exhibited significantly lower BBB scores than the sham group ($p < 0.01$), whereas eriodictyol treatment significantly elevated BBB scores compared to the untreated SCI group ($p < 0.01$) (Figure 1 A). In addition, SCI induction led to a decrease in PWT and PWL compared to the sham group ($p < 0.01$), whereas eriodictyol treatment increased both measures relative to the untreated SCI group ($p < 0.01$) (Figure 1 B and C). The spinal cord water content of SCI rats was increased compared to the sham group ($p < 0.01$), whereas eriodictyol administration significantly decreased spinal cord water content compared to the untreated SCI group ($p < 0.01$) (Figure 1 D).

Eriodictyol attenuates pro-inflammation cytokines in SCI rats

Levels of TNF-α, IL-1β, and IL-6 were significantly up-regulated in SCI rats compared to the sham group ($p < 0.01$). However, eriodictyol administration significantly inhibited pro-inflammatory cytokine up-regulation compared to the untreated SCI group ($p < 0.01$) (Figure 2).

Table 1: Primer pairs used for RT-qPCR

		Primer sequence
IL-6	forward	5′-CCA GAA ACC GCT ATG AAG TTCC-3′
	reverse	5′-TCA CCA GCA TCA GTC CCA AG-3′
TNF-α	forward	5′-CTC CAG GCG GTG CCT ATGT-3′
	reverse	5′-GAA GAG CGT GGT GGC CC-3′
IL-1β	forward	5′-CAA CCA ACA AGT GAT ATT CTC CATG-3′
	reverse	5′-GAT CCA CAC TCT CCA GCT GCA-3′

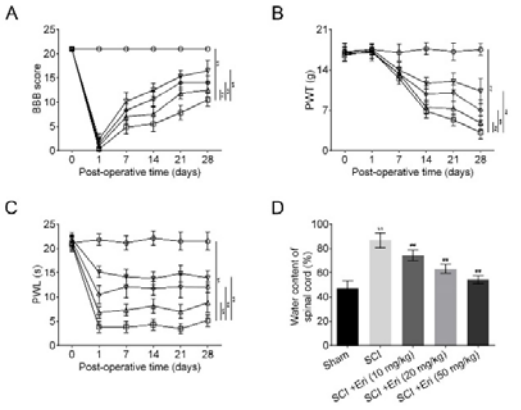

Figure 1: Eriodictyol alleviates motor dysfunction in SCI rats. (A) BBB scores in sham, SCI, and eriodictyol-treated groups; (B) PWT assessments in sham, SCI, and eriodictyol-treated groups; (C) PWL measurements in sham, SCI, and eriodictyol-treated groups; (D), Assessments of spinal cord water contents in sham, SCI, and eriodictyol-treated groups. ○, sham; □, SCI; △, SCI + Eri (10 mg/kg); ◇, SCI + Eri (20 mg/kg); ▽, SCI + Eri (50 mg/kg); $^{**}p < 0.01$, compared to the sham group; $^{\#\#}p < 0.01$, compared to the SCI group

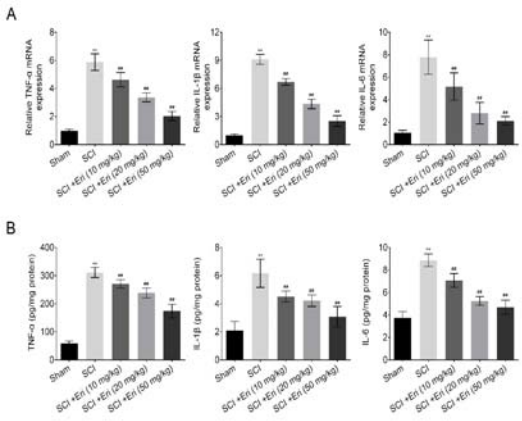

Figure 2: Eriodictyol attenuates pro-inflammatory cytokines in SCI rats. $^{**}p < 0.01$, compared to the sham group; $^{\#\#}p < 0.01$, compared to the SCI group

Eriodictyol attenuates oxidative stress in SCI rats

Induction of SCI led to increased MDA expression compared to the sham group ($p < 0.01$), whereas eriodictyol treatment significantly decreased MDA expression compared to untreated SCI rats ($p < 0.01$) (Figure 3 A). In contrast, SCI rats exhibited a significant decrease in SOD, GSH, and GSH-PX levels compared to the sham group ($p < 0.01$), whereas eriodictyol treatment gradually up-regulated SOD, GSH, and GSH-PX compared to the untreated SCI group ($p < 0.01$) (Figure 3 B - D).

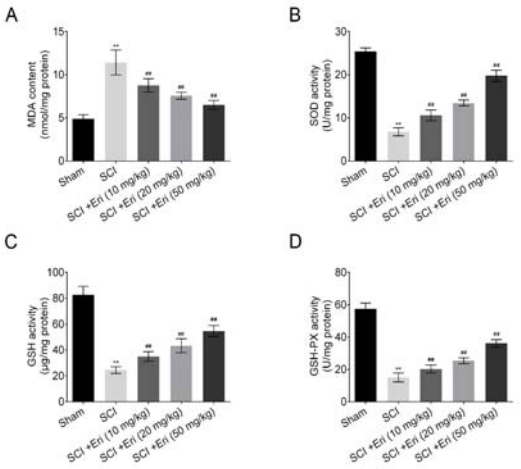

Figure 3: Eriodictyol inhibits oxidative stress in SCI rats. (A) MDA content in sham, SCI, and eriodictyol-treated groups; (B) SOD activity in sham, SCI, and eriodictyol-treated groups; (C) GSH activity in sham, SCI, and eriodictyol-treated groups; (D) GSH-PX activity in sham, SCI, and eriodictyol-treated groups; $^{**}p < 0.01$, compared to the sham group; $^{\#\#}p < 0.01$, compared to the SCI group

Eriodictyol regulates Nrf2/HO-1 and NF-κB pathways

Induction of SCI led to elevated Nrf2, HO-1, and p-NF-κB/NF-κB levels compared to the sham group ($p < 0.01$). Eriodictyol treatment (especially 20 and 50 mg/kg) significantly increased levels of Nrf2 and HO-1 ($p < 0.01$) but reduced the level of p-NF-κB/NF-κB compared to the untreated SCI group (Figure 4).

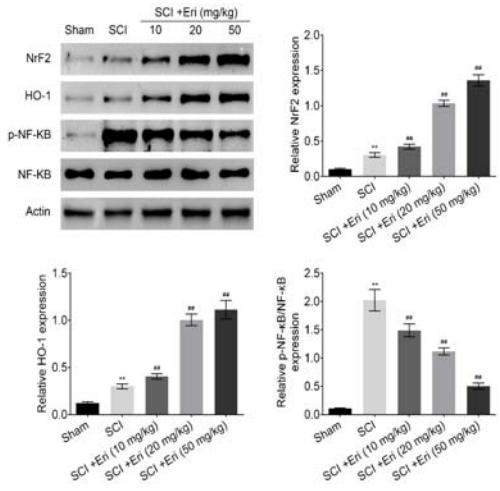

Figure 4: Eriodictyol regulates Nrf2/HO-1 and NF-κB signaling pathways in SCI rats. $^{**}P < 0.01$, compared to the sham group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared to the SCI group

DISCUSSION

Permanent disability and decreased quality of life and life expectancy can result from SCI [15]. Compelling evidence has shown that functional impairments following SCI result not only from the initial mechanical damage of the tissue but also from the development of complex secondary events that cause further cell damage [13]. Understanding how complex secondary injuries occur will facilitate the development of effective therapeutic strategy for patients with SCI. Therefore, this study investigated the protective effect of eriodictyol on SCI and its underlying mechanism. In this study, eriodictyol treatment alleviated locomotor dysfunction and decreased spinal cord water content following SCI. Eriodictyol treatment after SCI also reduced the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and attenuated oxidative stress by decreasing MDA and increasing SOD, GSH, and GSH-PX levels. Moreover, eriodictyol treatment after SCI increased the expression of Nrf2 and HO-1 and decreased p-NF-κB/NF-κB expression, indicating that eriodictyol may alleviate SCI via regulation of the Nrf2/HO-1 and NF-κB signaling pathways.

Inflammatory responses after trauma are likely to mediate early secondary injuries following SCI [13,16]. It has been reported that levels of IL-1β, IL-6, and TNF-α are remarkably elevated following severe SCI, reaching their highest levels at 6 h post-injury [17]. In an SCI mice model, significant up-regulation of TNF-α, IL-1β, and IL-6 were observed, whereas curcumin administration markedly inhibited the inflammatory response [18]. Oxidative stress and TNF-α, IL-1β, and IL-6 levels increased following SCI [19]. It has been reported that activation of the canonical NF-κB pathway following SCI indicates activation of the inflammatory response and that transplantation of neural precursors attenuates inflammation by inhibiting the NF-κB pathway [20]. Chlorogenic acid exerts anti-inflammatory effects via inactivating the Toll-like receptor-4/NF-κB and p38 pathways [21]. Consistent with these results, the present study demonstrated that SCI leads to increased levels of TNF-α, IL-1β, IL-6, and p-NF-κB/NF-κB relative to the sham group, whereas eriodictyol administration reduces TNF-α, IL-1β, IL-6, and p-NF-κB/NF-κB levels relative to the untreated SCI group. These results indicate that eriodictyol inhibits SCI-induced pro-inflammatory cytokines by suppressing the NF-κB pathway.

An earlier study showed that salvianolic acid A alleviates oxidative stress through activation of the Nrf2/HO-1 axis [22]. In a cisplatin-induced nephrotoxicity rat model, epigallocatechin-3-gallate increases antioxidant and GSH activates via activating the Nrf2/HO-1 pathway and reduces the inflammatory response by inhibiting NF-κB [23]. In traumatic brain injury mice, protein levels of IL-1β, IL6, and NF-κB decrease, whereas allyl isothiocyanate administration increases Nrf2 expression, indicating that oxidative stress and inflammation are alleviated via activating the Nrf2/HO-1 pathway or suppressing NF-κB pathway [24].

Tanshinone IIA treatment down-regulates MDA, elevates GSH levels, and attenuates oxidative stress via activating the DJ-1/Nrf2/HO-1 pathway [25]. Moreover, eriodictyol exerts a protective effect on endothelial cells by eliminating oxidative stress-induced cell death via regulation of extracellular signal-regulated kinase (ERK)/Nrf2/ARE-dependent HO-1 expression [26]. The present study found that eriodictyol treatment reduced MDA levels and increased SOD, GSH, and GSH-PX levels. Further, eriodictyol visibly increased Nrf2 and HO-1 levels, indicating that eriodictyol inhibits oxidative stress following SCI via activating the Nrf2/HO-1 pathway.

The results of this study show that eriodictyol exerts a protective effect on SCI in rats via modulation of the NrF2/HO-1 and NF-κB signaling pathways. Further research into the clinical potential of this compound should be conducted. In addition, the mechanism by which eriodictyol attenuates SCI through regulating Nrf2/HO-1 and NF-κB signaling pathways warrants future investigation.

CONCLUSION

The findings of this study demonstrate that eriodictyol alleviates SCI in rats by regulating Nrf2/HO-1 and NF-κB signaling pathways. Thus, the results support the need further investigation of eriodictyol as a potential therapeutic strategy for patients with SCI.

DECLARATIONS

Conflict of interest

No conflict of interest is 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.

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