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Original Article

Coenzyme Q10 suppresses oxidative stress and apoptosis via activating the Nrf-2/NQO-1 and NF-κB signaling pathway after spinal cord injury in rats

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Abstract: Spinal cord injury (SCI) is one of the most devastating diseases that may cause paralysis, disability and irreversible loss of functions, which ultimately lead to permanent disabilities and a decrease in patient life expectancy. Coenzyme Q10 (CoQ10) is a lipid-soluble vitamin-like benzoquinone compound that can exert antioxidant and anti-apoptotic functions in a variety of diseases. However, the antioxidant and anti-apoptotic effects of CoQ10 in the treatment of SCI are still unknown. Therefore, we designed experiments to measure the changes in antioxidant capacity (glutathione (GSH), superoxide dismutase (SOD) and the end product of lipid peroxidation (MDA)) and apoptosis products (Bax, Bcl-2 and Caspase-3) to evaluate the protective effects of CoQ10 on SCI and investigated whether CoQ10 exerts its functions through the Nrf-2/NQO-1 and NF-κB signaling pathway. Our results showed that CoQ10 treatment could significantly decrease the levels of oxidative products (MDA) and increase the activities of antioxidant enzymes (SOD and GSH) against oxidative stress, as well as decrease the levels of pro-apoptotic proteins (Bax and Caspase-3) and increase the levels of anti-apoptotic proteins (Bcl-2) against apoptosis after SCI. We also observed that CoQ10 exerted beneficial effects through the Nrf-2/NQO-1 and NF-κB signaling pathway. These findings suggested that CoQ10 had a protective effect by decreasing oxidative stress and apoptosis after SCI. Thus, our data may provide a new approach wherein CoQ10 may be considered as a potential effective therapeutic for the treatment of SCI.

Keywords: Spinal cord injury, coenzyme Q10, oxidative stress, apoptosis, Nrf-2

Introduction

Spinal cord injury (SCI) is one of the most devastating diseases that may cause paralysis, disability and irreversible loss of functions, which ultimately lead to permanent disabilities and decrease patient life expectancy [1-3]. Epidemiological data showed that approximately 23 cases per 1 million occurred every year in the world [4], and 17,000 new cases of SCI were investigated by the end of 2016 in the United States according to the study from the National Spinal Cord Injury Center website [5]. It not only affects the quality of life of patients but also places heavy burdens on families and society. Thus, controlling and improving the process of SCI may challenge both clinicians and scientists.

The pathological mechanism of SCI is complicated and includes primary and secondary damage [6, 7]. After irreversible primary damage, secondary damage such as oxidative stress and apoptosis play an important role in SCI [8, 9]. Oxidative stress results from the imbalance between antioxidant systems and reactive oxygen species [10]. Decreasing the levels of oxidative products such as the end product of lipid peroxidation (MDA) and increasing antioxidant enzymes (catalase, glutathione (GSH) and superoxide dismutase (SOD) can prevent tissue damage and improve the prognosis of SCI [11, 12]. Additionally, apoptosis, including the pro-apoptotic protein Bax, the anti-apoptotic protein Bcl-2 and Caspase-3, has also been reported to affect the extent

of neuronal tissue damage after SCI [13]. Downregulation of the expression of BAX and Caspase-3 proteins and upregulation of Bcl-2 protein expression can definitely reduce cell death and improve functional recovery after SCI [14, 15]. Therefore, blocking oxidative stress and apoptosis may be an effective therapeutic approach for SCI.

Coenzyme Q10 (CoQ10) is a lipid-soluble vitamin-like benzoquinone compound that plays an important role in the mitochondrial respiratory chain [16, 17]. Previous studies have shown that CoQ10 exerts its antioxidant and anti-apoptotic effects in a variety of diseases, such as heart [18], nervous system [19] and reproductive system diseases [20], and cancer [21]. However, the antioxidant and anti-apoptotic effects of CoQ10 in the treatment of SCI are still unknown.

Therefore, in the present study, we designed experiments to measure the changes in antioxidant capacity (GSH, SOD, and MDA), and apoptosis (Bax, Bcl-2, and Caspase-3) to evaluate the protective effects of CoQ10 on SCI and investigate whether CoQ10 exerts its functions through the Nrf2/NQO-1 and NF-κB signaling pathway.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats were purchased from Guangzhou University of Chinese Medicine (Guangzhou, China) and housed in a temperature-controlled environment (at room temperature of 23 ± 0.5°C, 35-55% humidity, and 12 h light/dark cycles) and supplied with standard rodent chow and water. All procedures were performed according to the animal guidelines of Guangzhou University of Chinese Medicine. The study was approved by the ethics committee of Guangzhou University of Chinese Medicine.

Experimental SCI model

Thirty adult male SD rats weighing 200 to 250 g were randomly divided into 3 groups (n = 10/group): 1) the sham group; 2) the SCI model group; and 3) the CoQ10 group. The SD rat SCI model was induced to a moderate contusion based on Allen's method as previously described [22]. Briefly, SD rats were anesthetized under sodium pentobarbital (40 mg/kg, i.p.), and then the incision area was shaved and a laminectomy was performed at the T9-T10 levels under sterile conditions. After exposing the spinal cord surface with intact dura, a 10-g weight impactor (diameter, 2 mm) dropped from a height of 50 mm onto the exposed dura at the T10 level (**Figure 1A**). The successfully induced SCI led to spinal cord congestion, tail swing reflexes, swaying legs and slow paralysis. Each rat was provided bladder pressing 3 times daily. CoQ10 (20 mg/kg, Shanghai Yuanye Biotechnology, Shanghai, China) was administered orally for 2 days before surgery and continued until sacrificing. Similar procedures and treatments were performed in all the vehicle groups. SD rats were sacrificed at 1 week postsurgery.

Function scale after SCI

The Basso, Beattie and Bresnahan (BBB) scale, which measures locomotor ability for 4 min according to the 21 different criteria for movement of the hindlimb, was applied to evaluate the functional recovery at 1, 3, 5 and 7 days post-SCI [23, 24]. Two trained investigators who were blinded to the experimental conditions analyzed the function scale, and the final result score was averaged for each rat.

Histopathological evaluation (HE) and Nissl staining

Paraffin sample sections (5 μm thickness) were deparaffinixed with xylene and then underwent HE and Nissl staining. A light microscope (Dialux 22, Leitz, Germany) was used to assess sections. Counting of damaged neurons was performed and the histopathological alteration of the gray matter was scored on a 6-point scale for HE staining: 0 = no observable lesion; 1 = gray matter contained 5-10 eosinophilic neurons; 3 = gray matter contained > 10 eosinophilic neurons; 4 ≤ 1/3 of the gray matter area infarction; 5 = 1/3-1/2 of the gray matter area infarction; 6 ≥ 1/2 of the gray matter area infarction [25]. Rexed's lamina system of gray matter was used to classify and count neurons for Nissl staining [9]. The pathological score for an individual animal was calculated as the average of all the sections from one spinal cord. All the histological examination was performed in a blind manner.

Determination of oxidative stress parameters (GSH, MDA and SOD)

According to the manufacturer's protocol of the GSH, MDA and SOD assays (Nanjing Jiancheng

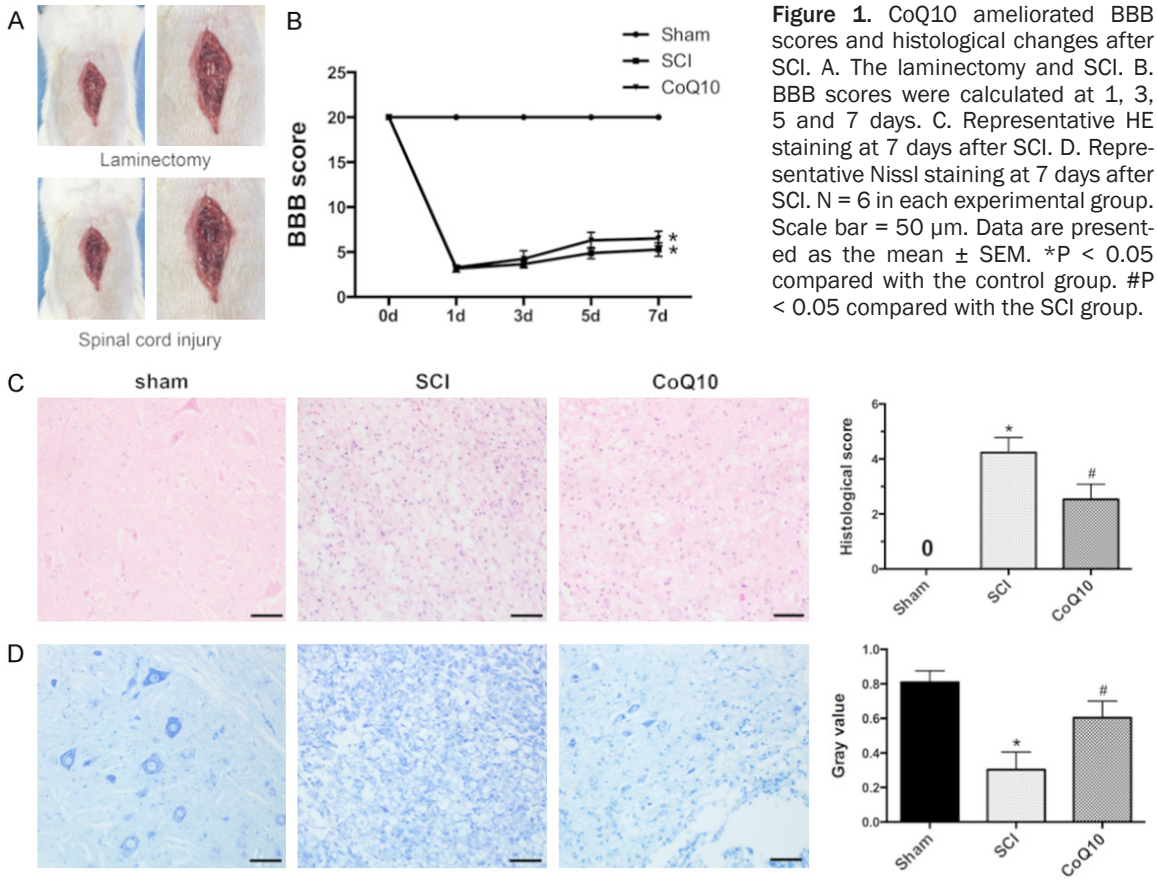

Figure 1. CoQ10 ameliorated BBB scores and histological changes after SCI. A. The laminectomy and SCI. B. BBB scores were calculated at 1, 3, 5 and 7 days. C. Representative HE staining at 7 days after SCI. D. Representative Nissl staining at 7 days after SCI. N = 6 in each experimental group. Scale bar = 50 μm. Data are presented as the mean ± SEM. *P < 0.05 compared with the control group. #P < 0.05 compared with the SCI group.

Bioengineering Institute), the liquid supernatant of spinal tissue samples was measured [26]. The GSH levels were analyzed at 420 nm, the MDA levels were determined at 532 nm, and the SOD activity was measured at 550 nm using a microplate reader (Bio-Rad, USA). All experiments were performed in triplicate.

TUNEL assay

The TUNEL assay was performed using a TUNEL cell apoptosis detection kit (Yeasen Biotech, Shanghai, China) according to the manufacturer's protocol [27]. The results were detected under fluorescence microscopy (Olympus DP80, Japan).

Western blot analysis

Western blot experiments were performed according to standard methods [28]. Briefly, the proteins were loaded in 10% SDS-PAGE gels and electrotransferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated with primary antibodies against Bax, Caspase-3 (1:1,000, Cell Signaling Technology), Bcl-2, NQO-1, p-p65, p65 (1:1000 dilution, Abcam, UK) and Nrf-2 (1:1,000, R&D) overnight at 4°C, followed by sequential incubation with the secondary antibodies conjugated with horseradish peroxidase (HRP) (1:1000, Cell Signaling Technology) at room temperature for 2 h. GAPDH (1:1000 dilution, Abcam, UK) was used as a reference. Quantified densitometry analysis was performed using an ImageQuant LAS 4000 mini detection system (GE Healthcare, Buckinghamshire, UK) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunohistochemical analysis

The rats were anesthetized and then perfused transcardially with ice-cold PBS followed by 4% PFA for 30 min. A 10-mm segment at the center lesion site of the spinal cord was collected for experiments. The samples were cut into serial crosswise sections of 4 μm using a cryostat microtome (Leica RM2016; Leica Instruments, Heidelberg, Germany). The spine tissues were treated with 3% hydrogen peroxide to block endogenous peroxidases and then incubated in 2% normal goat serum [29]. The sections

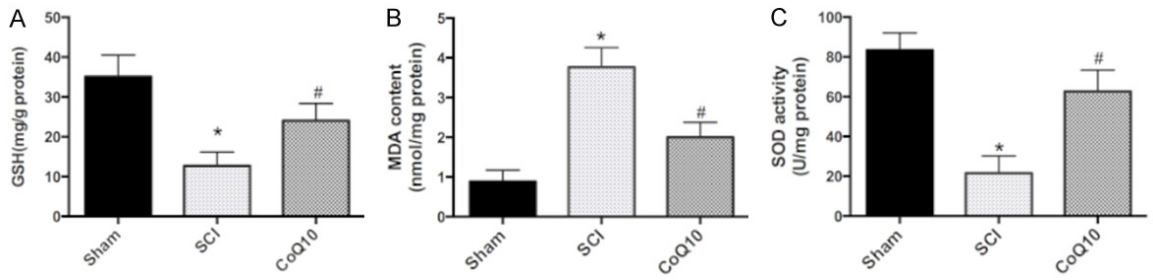

Figure 2. CoQ10 decreased oxidative stress in the rat SCI model. A. The level of GSH was measured using a GSH assay kit. B. MDA levels were measured using an MDA assay kit. C. The level of SOD was measured using an SOD assay kit. Data are presented as the mean ± SEM. *P < 0.05 compared with the control group, #P < 0.05 compared with the SCI group.

were incubated overnight with Caspase-3 (1:50 in PBS, Cell Signaling Technology) at 4°C. Sections were then washed with PBS and incubated with secondary antibodies. Specific labeling was detected with biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vector Laboratories, DBA). Images were photographed using fluorescence microscopy (Olympus DP80, Japan). Quantitative analysis of positive staining was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) or Student's t test was used to identify differences between groups. *P*-values of < 0.05 were considered statistically significant.

Results

Functional recovery

The BBB score was used to evaluate the functional recovery in post-SCI rats. As shown in **Figure 1B**, the BBB scores were significantly decreased in the SCI and SCI+CoQ10 groups at 1, 3, 5 and 7 days (P < 0.05). Although there was no statistically significant difference between the SCI and SCI+CoQ10 groups, CoQ10 treatment showed an escalating trend after SCI.

CoQ10 relieves histopathological damage after SCI

To explore the protective effects of CoQ10 on histopathological alterations, we examined the tissue histopathology using the HE and Nissl staining. The results of HE staining (**Figure 1C**) showed that the significant histopathological alterations were found after SCI (4.23 ± 0.6) compared to the sham group, including diffuse hemorrhage, widespread edema and congestion, neutrophil infiltration and neuronal disruption, and these changes were greatly attenuated under the treatment with CoQ10 (2.53 ± 0.5). In Nissl staining (**Figure 1D**), the expression of Nissl bodies were decreased in the in the SCI group (sham, 0.81 ± 0.06 versus SCI 0.30 ± 0.10), and CoQ10 treatment reversed the above result (CoQ10, 0.60 ± 0.10 versus SCI 0.30 ± 0.10). These results showed that treatment with CoQ10 significantly protected the histopathological alterations after SCI, although there was no statistically significant in functional recovery.

CoQ10 inhibits the antioxidative effects after SCI

To evaluate the protective effect of CoQ10 on oxidative stress following SCI, we measured the concentration of GSH, MDA and SOD in the liquid supernatant of spinal tissue samples. The results showed that the expression of GSH and SOD was decreased and the expression of MDA was increased in the SCI group, and the administration of CoQ10 had the ability to reverse the above results. These data indicated that treatment with CoQ10 significantly reduced oxidative stress after SCI. (**Figure 2A-C**).

CoQ10 suppressed apoptosis after SCI

To investigate the effects of CoQ10 on apoptosis after SCI, downregulation of the anti-apoptotic protein Bcl-2 and upregulation of the pro-

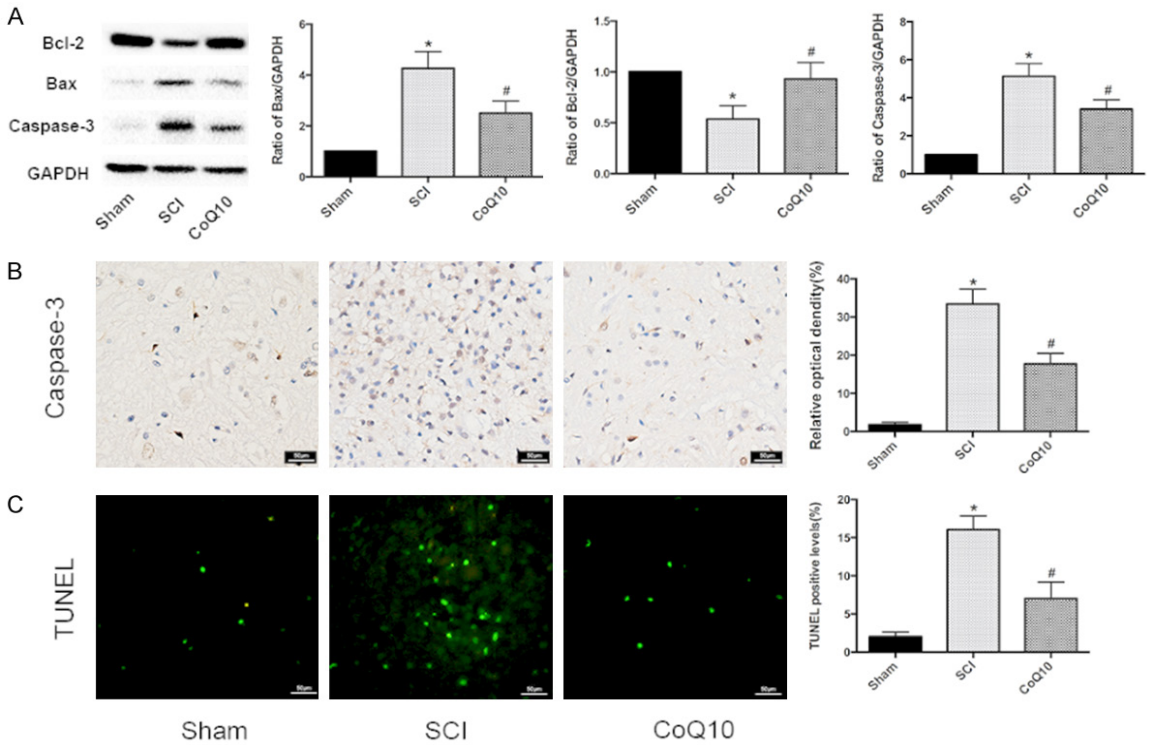

Figure 3. CoQ10 reduced apoptosis in the rat SCI model. A. The protein expression of Bax, Bcl-2 and Caspase-3 in spine tissue samples was measured by western blot. B. Immunohistochemical analysis of Caspase-3 protein expression. C. TUNEL staining of the cell apoptosis rate. Data are presented as the mean ± SEM. *P < 0.05 compared with the control group, #P < 0.05 compared with the SCI group.

apoptotic proteins Bax and Caspase-3 were measured in the spine tissue samples. As shown in **Figure 3A**, the expression of Bcl-2 protein was decreased and the expression of Bax and Caspase-3 proteins was increased in the SCI group by western blot, and with the CoQ10 treatment, the opposite results were shown by western blot. Moreover, according to the immunohistochemistry analysis (**Figure 3B**), we also found consistent results for Caspase-3 protein. Furthermore, TUNEL staining, which was used to evaluate the cell apoptosis rate, showed that the cell apoptosis rate was increased markedly in the SCI group and decreased significantly after treatment with CoQ10 (**Figure 3C**). These findings illustrated that treatment with CoQ10 significantly reduced apoptosis after SCI.

CoQ10 exerted antioxidative and anti-apoptosis effects through the Nrf-2/NOQ-1 and NF-κB signaling pathway

To explore whether the Nrf-2/NOQ-1 and NF-κB signaling pathway was involved in the protection of CoQ10 against oxidative stress and apoptosis, the proteins Nrf-2, NOQ-1, p-p65 and p65 were measured in the spine tissue samples by western blotting. The results showed that the protein levels of Nrf-2 and NOQ-1 were significantly decreased and the protein levels of p-p65 was significantly increased in the SCI group, after administration of CoQ10, these effects were significantly reversed (**Figure 4**). These findings indicated that the antioxidative and anti-apoptotic effects of CoQ10 on SCI occurred through the Nrf-2/NQO-1 and NF-κB signaling pathway.

Discussion

SCI leads to catastrophic dysfunction and a low mortality rate and remains a devastating problem for patients worldwide. Ameliorating and reducing the damage after SCI is considered one of the greatest challenges for basic science research and clinical treatment [30]. In the present study, we demonstrated that CoQ10 treatment could significantly protected the histopathological alterations, decrease the levels of oxidative products (MDA) and increase the activities of antioxidant enzymes (SOD and GSH) against oxidative stress, while also

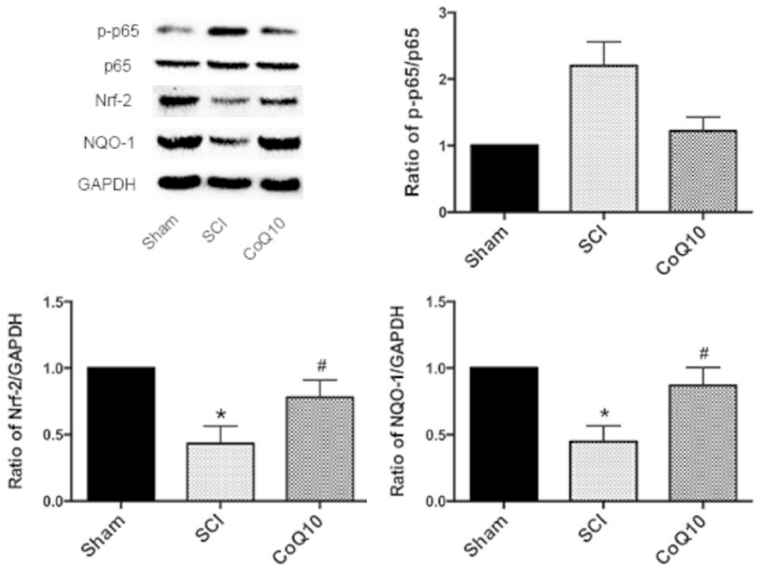

Figure 4. CoQ10 activated the Nrf-2/NQO-1 and NF-κB signaling pathway after SCI. The protein expression of Nrf-2, NQO-1, p-p65 and p65 in spine tissue samples were measured by western blot. Data are presented as the mean ± SEM. *P < 0.05 compared with the control group, #P < 0.05 compared with the SCI group.

decreasing the level of pro-apoptotic proteins (Bax and Caspase-3) and increasing the level of anti-apoptotic proteins (Bcl-2) against apoptosis after SCI. We also observed that CoQ10 exerted beneficial effects through the Nrf-2/NQO-1 and NF-κB signaling pathway. These findings suggested that CoQ10 had a protective effect by decreasing oxidative stress and apoptosis after SCI.

Oxidative stress is a severe pathological process and plays an important role during SCI [6, 31, 32]. During this process, the balance between pro-oxidants and antioxidants is disrupted. MDA, the ultimate product of lipid peroxidation, provides a reliable marker for peroxidation and increases during stress [33]. GSH and SOD are important endogenous antioxidants [34] that can scavenge free radicals to exert antioxidant functions [35]. Consistent with previous research [9, 36], increased expression of MDA and decreased expression of GSH and SOD were shown in the SCI group. However, CoQ10 treatment significantly decreased the expression of MDA and increased the expression of GSH and SOD compared with levels in the SCI group. These data showed that CoQ10 had antioxidant ability.

Apoptosis, a process of programmed cell death, plays a pivotal role during SCI [37]. The Bcl-2 family, including pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins, is a main factor regulating apoptosis. Bax showed an ability to increase cytochrome c release and result in caspase-induced apoptosis [38]. However, Bcl-2, when combined with Bax to form a heterodimer, inhibited apoptosis [38]. In addition, Caspase-3, known as the key executioner caspase [39], could activate the DNA fragmentation factor and ultimately resulted in cell apoptosis [40, 41]. Thus, the expression of Bax, Bcl-2 and Caspase-3 absolutely influenced the progression of apoptosis. In our study, CoQ10 treatment significantly decreased the levels of Bax and Caspase-3 and increased the level of Bcl-2 compared with those in the SCI group. Moreover, the same trend was observed in the immunohistochemical analysis and TUNEL staining. Therefore, the present study suggested that CoQ10 had a beneficial effect against apoptosis in SCI.

Many studies have reported that nuclear factor erythroid 2-related factor 2 (Nrf2) plays an important role in protecting against oxidative stress [42]. Nrf2, a basic-leucine zipper transcription factor, leads to antioxidant and detoxifying gene modulation [43]. Under stress conditions, Nrf-2 translocates to the nucleus, binds to antioxidant response element (ARE) and activates cytoprotective proteins and the transcription of genes encoding detoxifying enzymes, such as quinone oxidoreductase 1 (NQO1) and superoxide dismutase, which lead to oxidative stress and apoptosis after SCI [44-46]. Moreover, our previous researches also demonstrated that the activating the Nrf-2/NQO-1 signaling pathway had the ability to reduce the oxidative stress products and ROS [26, 47]. NF-κB, as one of the cytokine-induced transcription factors, could be widely activated by stress, free radicals and ROS [48, 49]. NF-κB exists in the cytoplasm and forms of a complex with IκB in the normal cells. When stimulated by the stress, free radicals and ROS, NF-κB is activated, which leads to subsequent phosphorylation of p65. The phosphorylated p65

will be translocated from cytosol to nucleus and activates the ROS, eventually resulting the oxidative stress. In the present study, treatment with CoQ10 significantly increased the levels of Nrf-2, NQO-1 protein and decreased the expression of p-p65 compared with the SCI group. These results indicated that CoQ10 exerted antioxidant and anti-apoptotic functions after SCI via the Nrf-2/NQO-1 and NF-κB signaling pathway.

In conclusion, our results showed that CoQ10 significantly decreased oxidative stress and apoptosis after SCI and exerted antioxidant and anti-apoptotic functions via the Nrf-2/NQO-1 and NF-κB signaling pathway. These results may provide a new approach wherein CoQ10 may be considered as a potential effective therapeutic for the treatment of SCI.

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Disclosure of conflict of interest

None.

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