

Effect of Electroacupuncture on Spinal Cord Injury in Mice: Inhibition Of Inflammatory Response and Oxidative Stress via ApoE and Nrf2

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Research

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Abstract

Background: Spinal cord injury (SCI) is a catastrophic central nervous system disease. Inflammatory response and oxidative stress are two critical factors in the pathophysiological process of SCI and closely involved with Apolipoprotein E(ApoE) and Nuclear factor erythroid 2-related factor (Nrf2).

Electroacupuncture (EA) has perfectly neuroprotective effect on SCI. However, the underlying mechanism by which EA mediates the inflammatory response and oxidative stress is not completely elucidated. In the present study, we investigated the signaling pathways that EA regulates inflammatory response and oxidative stress through elevation of ApoE and Nrf2 after SCI.

Methods: C57BL/6 Wide Type (WT) mice and ApoE ^{-/-} mice were subjected to SCI model by a serrefine clamping. Neurological function was detected by BMS scores, ultrastructure of demyelinated axons was observed by transmission electron microscopy. ApoE, pro- and anti-inflammatory cytokines, oxidative stress-relevant proteins were determined by histochemistry technology. Two-way ANOVA was applied to BMS scores. One-way ANOVA and Bonferroni's multiple comparison test were used to analyse differences among groups.

Results: BMS scores were increased gradually and demyelinated axons were improved by EA gradually with the expression of ApoE. EA can inhibit inflammatory response by activation of ApoE, which decreased pro-inflammatory cytokines(TNF- α , IL-6, and IL-1 β) expression and increased anti-inflammatory cytokines(IL-10 and TGF- β 1).Meanwhile, EA can also inhibit oxidative stress by elevation of Nrf2,which induced HO-1 and NQO1 expression in WT and ApoE ^{-/-} mice.

Conclusions: EA is a reliable treatment for promoting functional recovery of SCI. The synergistic role of ApoE and Nrf2 in EA regulating inflammatory response and oxidative stress is decisive to recovery after SCI.

Background

Spinal cord injury (SCI) results in sensory, motor and autonomic impairments below the injury level, and now there are no effective treatments for this neurological disorder[1, 2]. It is increasingly becoming a global medical problem because of its substantial individual and societal economic burden[3]. Previous studies have demonstrated that secondary damages, including inflammatory response, oxidative stress, neuronal apoptosis, axonal demyelination, etc., occur further on the basis of primary injury, leading to irreversible damage to the spinal cord[4, 5]. Notably, inflammation response and oxidative stress are the critical factors in the secondary injury and closely involved with the pathophysiological mechanisms of SCI[6-8]. Thus, how to inhibit inflammation response and oxidative stress might be an effective neuroprotective strategy.

Electroacupuncture (EA) is widely used in various acute and chronic diseases, and has shown a good curative effect on central nervous system (CNS) diseases, especially SCI[9-12]. Several reports show that EA could effectively facilitate the cell survival and reduce the neurons apoptosis[13-15], prompt neural

stem cell or precursor cells to proliferate and differentiate into neurons[16, 17], accelerate axonal regeneration and remyelination[18, 19] after SCI. In particular, recent reports have shown that EA could alleviate inflammatory response via inhibiting the activation of NF- κ B signaling pathway or microglia cells[20, 21], or downregulating the M1 macrophages markers as well as upregulating the M2 macrophages markers[22]. EA can also control oxidative stress by reducing the amount of reactive species and increase the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase in experimental rat models[23]. Although extensive studies on EA were related to the mechanisms of neurological diseases, the role of EA in inflammatory response and oxidative stress after SCI has not been well investigated.

Apolipoprotein E(ApoE) is primarily expressed in three isoforms (ApoE2, ApoE3, and ApoE4) that differ only by two residues[24]. It is a major apolipoprotein for the regulation of lipid and cholesterol metabolism in CNS[25-27]. But now it is closely associated with physiopathology of many neurological diseases such as Alzheimer's[28]and atherosclerosis[29].For example, ApoE 4 is high risk factor to the early onset of Alzheimer's disease[24] and rapid progression of multiple sclerosis[30]. ApoE 4 is also associated with cerebral ischemia[31] and poor neurological outcome following stroke[32]. While ApoE3 is neutral, and ApoE2 is protective[24]. Interestingly, ApoE possesses the properties of anti-inflammatory, anti-oxidative stress, anti-apoptosis in the CNS disease[33-35].Deficiency of ApoE results in exaggerated inflammatory response[36], reduces antioxidants levels [37], and prompts neuronal apoptosis after SCI[36] .Conversely, treatment with exogenous ApoE can ameliorate motor deficit and tissue damages and modify the inflammatory response in CNS [38, 39].So there is a widely accepted statement that ApoE plays novel role in neuroprotective function after neurological diseases[2]. Nuclear factor erythroid 2-related factor (Nrf2), encoded by the NFE2L2 gene, is considered not only a transcription factor for cellular redox balance, but also cytoprotective factor for anti-oxidation, anti-inflammation[40-42].Nrf2 activation is neuroprotective in neurodegenerative diseases such as Parkinson disease and multiple sclerosis[43]; impaired Nrf2 signaling may lead to oxidative stress in Friedreich ataxia[44] .Some antioxidant response elements, including heme oxygenase-1 (HO-1)[45], and NAD(P)H-quinone oxidoreductase 1 (NQO1) are regulated through Nrf2 binding to the consensus binding sequence[46]. HO-1, one of two distinct HO isoforms found in mammals[40], provides cytoprotective effect and plays a crucial role in the development of oxidative and age-related disorders[40]. The level of HO-1 decreases in the mouse spinal cord with aging[47]. NQO1 plays an important role in neuroprotection through its anti-oxidative properties as well. Activation of Nrf2 signaling leads to an elevation of NQO1[48]. Several studies showed that activation of Nrf2/HO-1/NQO1 affects oxidative status of the cells, provides robust protection against oxidative challenge, and participates in pathogenesis and therapy of stress activated and age-related diseases in the end[40, 49, 50].

Given that the data suggested the effects of acupuncture on neurological diseases and the role of ApoE and Nrf2 in the inflammatory response and oxidative stress, in the study, we hypothesized that EA would inhibit the inflammatory response and oxidative stress through the synergy of ApoE and Nrf2 after SCI and provide important insights into the treatment of SCI.

Methods

Surgical Procedure

Adult (2–3 months old) female C57BL/6 Wild Type (WT) mice and homologous ApoE^{-/-} mice weighing 18 - 20 g were used in this experiment. Animals were obtained from the Experimental Animal Center of Chongqing Medical University and housed in the standard cage in the Specific Pathogen Free (SPF) grade room with a 12-hour light/dark cycle, room temperature 22 - 24°C, relative humidity 65% - 75% and free access to food and water. All experiments were carried out in strict accordance with *Guiding Opinions on the Treatment of Experimental Animals* promulgated by the Ministry of Science and Technology of the People's Republic of China in 2006. Disposal of animals throughout this experiment complied with the standards of the Ethics Committee of Chongqing Medical University.

WT mice and ApoE^{-/-} mice were anesthetized by intraperitoneally injecting 1% sodium pentobarbital (80 mg/kg, i.m.). Throughout the surgery, the mice kept deep sedation. A sterile and limited laminectomy was performed at the first lumbar vertebra without disrupting the dura. The exposed dorsal surface of the cord was subjected to clamping injury with a symptom of a spasmodic tail and tremulous hindlimbs by a serrefine for 25s [51]. Heating facility was supplied to mice until they fully recovered from the anesthesia. After surgery, muscles and skins were sutured in layers. Penicillin (40,000 U, i.m.) had been applied for 3 days to prevent infection. Abdominal massage was implemented twice daily to improve bladder dysfunction until mice resumed spontaneous micturition.

All the mice subjected to same degree injury were selected into the study. WT mice were divided into: sham group, control group, EA group, EA+COG112 (ApoE mimetic peptides administration) group. ApoE^{-/-} mice were divided into: EA group, EA+COG112 group, with 18 mice in each group. In addition to postoperative basic care, different groups of mice received the following treatment: the sham group underwent only laminectomy on L1 without damaging the spinal cord; the control group received no treatment but free access to food and water; the EA group received EA treatment; the EA+COG112 group received the combination of EA treatment and COG112 treatment.

EA Treatment

In the EA and EA+COG112 groups, mice received EA treatment. EA stimulation was performed at the bilateral “Zusanli” (ST 36, located at the anterior aspect of the hindlimb, straightly 2 mm below the knee joint) and “Sanyinjiao” (SP 6, located at the posterior to the tibia, 3 mm above the medial malleolus). Stainless steel needles of 0.25 mm × 13 mm were inserted to a depth of 3-5 mm at each acupoint. Then, the needle handles were linked to the output terminals of an electronic acupuncture instrument (SDZ - II, Suzhou Medical Products Co., Ltd., China). An intermittent wave was used to stimulate acupoints, and the hind limbs showed a mild twitch. The intensity lasted 10 min every time for each. EA had been administered for 4 weeks (six days for continuous treatment followed by one day off per week), starting from the first day post-surgery.

COG112 preparation and application

COG112, one of ApoE mimetic peptides with anti-inflammation character used in this experiment, was synthesized by the laboratory (Gill Biochemical Co., Ltd., Shanghai, China). The peptide sequence of COG112 is acetyl-RQIKIWFQNRRMKWKKCLRVLASHLRKLRKRL-amide. All peptides were purified by high-performance liquid chromatography (HPLC) with a purity of >95%.

In the COG112 and EA+COG112 groups, mice received COG112 treatment. COG112 was formulated with lactated ringer's buffer and the single dose of each mouse was 1 mg/kg, twice daily for 4 weeks. The first injection was performed at 3 h after surgery by intraperitoneal injection.

Behavior test

Basso Mouse Scale (BMS), an effective scoring method for injured animals to assess the hind limb locomotor function, ranges from 0 to 9, in which 0 denotes complete paralysis and 9 denotes normal movements. Two raters who were familiar to the scoring criteria and blinded to this experiment scored the hind limb locomotor function of mice according to the ankle joint mobility, coordination, paw status, trunk stability and tail posture. Each mouse was observed for 5 min in an open field, and the average of two raters'scores was taken as the final score. This test was performed on day 1, 3, 7, 14 and 28 after SCI, and only mice scored 0 - 0.5 were applied to this experiment.

Tissue Preparation

On the 28th after SCI, mice were deeply anesthetized and transcardially perfused with 0.01M phosphate buffered saline (PBS), followed by 4% paraformaldehyde solution (4% in 0.01 PBS) at room temperature. After fixated with the universal tissue fixative (Google Biotech Co., Ltd., Wuhan, China) for 24 h, the tissue was completely dehydrated with graded ethanol, permeabilized with xylene, embedded in paraffin and cut into 6 μ m thick sections for histological analysis.

Hematoxylin/eosin (HE) staining

Tissue sections were deparaffinized with xylene and, and immersed in hematoxylin solution for 3 min, following by rinsing quickly in distilled water. Then the slides were differentiated in HCL/95% alcohol (1:50) solution for 10 s. After washing in distilled water for 5 min, the sections were stained by 0.5% eosin for 10 s, dehydrated by gradient ethanol for 3 min (95%, 100%), and then transparentized by xylene for 1 min. The sections were examined under a fluorescent microscope (BX 53, Olympus, Japan). The pathological scores were determined by edema, hemorrhage, tissue cavity, neuronal apoptosis or necrosis, and inflammatory cells infiltration and represented the degree of SCI over a range from 0 to 4 points: 0 for none or minor, 1 for limited, 2 for intermediate, 3 for prominent, and 4 for widespread. Two raters who were familiar to the scoring criteria and blinded to this experiment scored pathological changes.

Immunofluorescence (IF)

For immunofluorescence staining, Tissue sections were deparaffinized by xylene and rinsed, blocked with goat serum (Boster, China) at 37 °C in a humidified atmosphere for 1 h, and incubated with primary antibodies, including rabbit anti-ApoE (1:400, Abcam, America), rabbit anti-Nrf2 (1: 400, Abcam, America) overnight at 4 °C. The sections were rinsed again with PBS and incubated with secondary antibodies (goat IgG conjugated with Alex 492, 1:200, Earthox, Shanghai) for 1 h at 37 °C in a humidified atmosphere in the dark. Nuclear dye (4',6-diamidino-2-phenylindole, Beyotime Bio, C1005, China) was applied to sections for 5 min. Then, the sections were rinsed again and mounted with an antifade mounting medium (Beyotime Bio, China). The samples were observed under a fluorescence microscope.

Transmission Electron Microscopy (TEM)

Mice were deeply anaesthetized and transcardially perfused with PBS followed by a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde (1:1). The spinal cord containing the lesion epicenter (5 mm) was removed and fixed for overnight at 4°C. Samples were placed in 1% osmium tetroxide and dehydrated in ascending graded ethanol and acetone. Following embedding in Epon-Araldite resin, tissues were cut into ultrathin sections using a reichert ultramicrotome and then collected with copper grids. After staining with uranyl acetate and lead citrate, the sections were photographed by a Philips EM420 electron microscope. Image Pro Plus software was used to quantify the ratio of demyelinated axons to total axons.

Western blot (WB) analysis

On the 28th after SCI, mice were deeply anaesthetized and transcardially perfused with 0.01 M PBS. Spinal segments (1cm) containing the lesion were immediately removed and grounded on ice in a mixture of RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing 1% protease inhibitor. The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was diluted with 5× protein loading buffer and then heated at 95°C for 5 min. Proteins in samples containing the same mass of protein (40 µg) were separated by the 10% SDS polyacrylamide gel electrophoresis system, and then transferred to PVDF membrane. After being blocked the non-specific binding site with 5% skim milk for 2 h at room temperature, the membranes were incubated with the following antibodies at 4 °C for overnight: rabbit anti-β-actin (1:5000, CST, USA) , rabbit anti-Nrf2 (1: 1000, Abcam, America), rabbit anti-HO-1 (1:2000, Abcam, America), rabbit anti-NQO1 (1:10000, Abcam, America), rabbit anti-ApoE (1:1000, Abcam, America). Horseradish peroxidase-labeled goat anti-rabbit IgG (1:100; Boster) was then incubated for 1 h at 37 °C. Protein bands were exposed for 1 minute with Enhanced Chemiluminescence (ECL) detection reagent (Applygen Technologies Inc., Beijing, China). The intensity of all protein bands was measured by Image J Software and the relative band intensities of the target proteins were normalized to those of the respective β-actin internal controls. All western blot experiments were performed in triplicate.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The mRNA expression levels of pro-and anti-inflammatory cytokines were determined at 28 dpi. Mice were transcardially perfused with 0.01M PBS as mentioned earlier. Standardized areas of spinal cord

tissue (5 mm cephalad and 5 mm caudal to the lesion center) were harvested. Total mRNA was extracted using the RNAiso Plus (TaKaRa, Japan), according to the manufacturer's instructions. Reverse transcription to cDNA was performed following the reaction protocol provided with the PrimeScript® RT reagent Kit With gDNA Eraser. Quantitative PCR was conducted by the CFX 96 real-time PCR detection system (Bio Rad, America) under universal cycling conditions (30 s at 95°C followed by 40 cycles of 5 s at 95°C, and 30 s at 60°C). The reaction mixture contained fast SYBR Green master mix (Applied TaKaRa), 10mM forward and reverse primers (TaKaRa), RNase free water, and cDNA in a total reaction volume of 10 µl. Relative quantification of gene expression was accomplished by using the $2^{-\Delta\Delta CT}$ method and data were normalized to the most stable reference genes. The primer sequences used are shown in Table 1.

Table 1 Forward and reverse primer sequences for qRT-PCR

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
TNF- α	FP: 5'-GCCAACGGCATGGATCTCAA-3'	RP: 5'-TGACGGCAGAGAGGAGGTTGA-3'
IL-6	FP: 5'-CTTGGGACTGATGCTGGTGAC-3'	RP: 5'-TTCTCATTCCACGATTCCCA-3'
IL-1 β	FP: 5'-GCTGCTTCCAAACCTTTGACC-3'	RP: 5'-AATGAGTGATACTGCCTGCCTGA-3'
IL-10	FP: 5'-TTGCCAAGCCTTATCGGAAAT-3'	RP: 5'-TGAGGGTCTTCAGCTTCTCACC-3'
TGF- β 1	FP: 5'-GAGGCGGTGCTCGCTTTGTA-3'	RP: 5'-CGTTGTTGCGGTCCACCATTA-3'
β -actin	FP: 5'-AGATTACTGCTCTGGCTCCTAGC-3'	RP: 5'-ACTCATCGTACTCCTGCTTGCT-3'

Statistics

All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, USA), in which data were expressed as Mean \pm standard error of the mean (SEM) and analyzed through ANOVA. $P < 0.05$ denoted statistical significance. Two-way ANOVA was applied to BMS scores. One-way ANOVA and Bonferroni's multiple comparison test were used to analyse differences among groups. At 95% confidence interval, differences were considered statistically significant when $P < 0.05$.

Results

EA promotes functional and morphological recovery after SCI in WT mice

To investigate the effects of EA on functional and morphological recovery after SCI, firstly, BMS was performed to assess the locomotor function on 1, 3, 7, 14 and 28 dpi in WT mice (Fig. 1A). The WT sham group displayed normal functional outcomes. No statistically significant differences were found at 1dpi between the WT control and EA groups ($p > 0.05$), the mean BMS scores in which were increased with the

duration of time. However, BMS scores in the WT EA group was significantly higher compared with that in the WT control group, especially on 28 dpi after SCI (Fig. 1B). Secondly, HE staining was used to observe the histomorphology of the spinal cord near the epicenter on 28 dpi. In the WT sham group, the spinal cord structure is normal. After injury, significant damage to the spinal cord occurred and manifested as structural disorganization, tissue cavities, neuronal apoptosis or necrosis and inflammatory infiltration in both the WT control and WT EA groups, while the damaged spinal cord was significantly improved after EA treatment when compared to the WT control group. The pathological score in the WT EA group was significantly lower than that in the WT control group (Fig. 1C, D). Thirdly, myelination is the material basis for rapid nerve impulse conduction[52], so we observed the ultrastructure of axons and myelin sheaths in the spinal cord by TEM. In the WT sham group, the axons were tightly wrapped by the compact myelin sheaths in layers. In contrast, a mass of myelin sheaths significantly degenerated into loose and separated from the axons (named demyelinated axons) after injury. The ultrastructural features of demyelinated axons were significantly enhanced by EA compared to the WT control group and the ratio of demyelinated axons to total axons in the WT EA group was significantly lower than that of the WT control group (Fig. 1E, F).

Taken together, compared with the WT control group, EA significantly enhanced hindlimb locomotion function, reduced neural tissue loss and inflammatory response, and suppressed myelin degeneration and axonal demyelination, which suggested that EA played a critical role in promoting spinal cord recovery after injury.

EA increases the expression levels of ApoE and Nrf2 after SCI in WT mice

To assess the effects of EA on ApoE after SCI, we examined the expression level of ApoE by western blot and IF staining. Given that Nrf2 possesses the same anti-inflammatory, antioxidant and anti-apoptotic properties as ApoE, we detected the expression level of Nrf2 as well. The protein expression levels of ApoE and Nrf2 in the spinal cord of WT control and WT EA groups were significantly increased on day 0 compared with the WT sham group, and there was no significant difference between the control and EA groups. After 28 days, the protein expression levels of ApoE and Nrf2 in the WT control and WT EA groups were both significantly further increased, and the increase in the WT EA group was significantly superior to that in the WT control group (Fig. 2A-C). IF staining showed the number of ApoE and Nrf2 positive cells in the spinal cord was small under normal circumstances. Nevertheless, the number of them began to elevate following injury, and significantly further increased with EA treatment (Fig. 2D-F). It suggested that EA can increase the expression levels of ApoE and Nrf2 after SCI.

Effects of EA on functional and morphological recovery after SCI depend on the expression of ApoE

To explore whether ApoE is a target of EA to promote the recovery from SCI, we observed the expression of ApoE on day 28 after SCI in WT and ApoE^{-/-} mice by western blot and IF staining firstly. As a whole, the protein expression levels of ApoE are decreased gradually in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups respectively, and there was no expression of ApoE in the ApoE^{-/-} EA

group, the difference were significant ($p < 0.001$, $p < 0.01$, Fig, 3A, B). These findings showed that the application of COG112 effectively increased the expression of ApoE. The results of IF staining presented the same trend as that in western blot analysis (Fig, 3C, D). Secondly, the effects of EA on functional and morphological recovery after SCI were detected when the expression of ApoE were different in mice.

BMS was performed to assess the locomotor function of the hind limb on day 1, 3, 7, 14 and 28 after SCI in WT and ApoE^{-/-} mice. As a whole, the hindlimb locomotions of mice in the four groups were improved as time goes by, and presented significant differences among the four groups on day 7, 14 and 28 after SCI (Fig. 4A), especially on day 28. We found that the BMS scores in WT mice were higher than those in ApoE^{-/-} mice, BMS scores were decreased gradually when the expressions of ApoE were reduced in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups respectively, the difference were significant ($p < 0.001$, $p < 0.01$, Fig. 4B). It indicated that EA can improve the locomotor function of mice after SCI and the effect of EA on the locomotor function depends on the ApoE expression in the mice.

HE staining and TEM were used to observe the histomorphology and the ultrastructure of the spinal cord near the epicenter on day 28 after SCI. The results showed neural tissue loss, inflammatory response, myelin degeneration and axonal demyelination occurred in the four groups (Fig. 4C, D). However, only can the pathological damages be improved by EA when ApoE was involved. In addition, pathological score and the ratio of demyelinated axons to total axons were increased gradually when the expressions of ApoE reduced in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups respectively, the difference were significant ($p < 0.001$, $p < 0.01$, Fig. 4E, F). Taken together, we found that the deficiency or low-expression of ApoE significantly inhibited the effects of EA on spinal cord recovery after SCI. Conversely, the application of exogenous ApoE significantly strengthened the effects of EA. It suggested that EA ameliorated SCI in an ApoE-dependent manner. In other words, ApoE is one of the targets of EA to promote the recovery of SCI.

Effects of EA on inflammatory response after SCI depend on the expression of ApoE

TNF- α , IL-1 β , IL-6, IL-10 and TGF- β 1 have been reported as inflammatory related cytokines in a variety of diseases, and play an important role in exacerbating or hindering inflammatory response[53–56]. To investigate whether the effects of EA on inflammation response after SCI were closely related to ApoE, we detected the mRNA levels of TNF- α , IL-1 β , IL-6, IL-10 and TGF- β 1 in WT and ApoE^{-/-} mice. The results showed that the mRNA levels of pro-inflammation cytokines (including IL-1 β , IL-6 and IL-10) were increased gradually when the expressions of ApoE were reduced in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups respectively, the difference were significant ($p < 0.001$, $p < 0.01$. Figure 5A). While the mRNA levels of anti-inflammation cytokines (including IL-10 and TGF- β 1) were decreased gradually when the expressions of ApoE were reduced in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups, respectively. The difference was significant ($p < 0.001$, $p < 0.01$. Figure 5B). It suggested that the effect of EA on inhibiting inflammatory response is closely involved with ApoE as well.

Effects of EA on oxidative stress after SCI depend on the expression of ApoE

In addition to inflammatory response, we also investigated the effects of EA on oxidative stress after SCI through exploring the levels of Nrf2/HO-1 signaling pathway. The results showed that the expression levels of Nrf2/HO-1 signaling pathway including Nrf2, HO-1 and NQO1 were highly consistent with the expression of ApoE. In detail, the expression levels of Nrf2, HO-1 and NQO1 were decreased gradually when the expressions of ApoE were reduced in the WT EA + COG112, WT EA, ApoE^{-/-} EA + COG112 and ApoE^{-/-} EA groups respectively, the difference were significant ($p < 0.001$, $p < 0.01$. Fig. 6A, B). It suggested that effects of EA on oxidative stress were closely related to the Nrf2/HO-1 signaling pathway which depended on the expression of ApoE.

Discussion

In the present study, based on analyses of ApoE knockout and WT mice with SCI, our data indicated that EA improved locomotor dysfunction, reduced inflammatory response and oxidative stress, and enhanced remyelination after SCI in WT mice. While in ApoE^{-/-} mice, the neuroprotective effect of EA on SCI was inhibited. However, the supplement of exogenous ApoE (COG112) made the EA effect renewed, suggesting that ApoE is an important therapeutic target of EA on SCI and the effect of EA depends on the ApoE expression. In addition, we provide clear evidence that EA can inhibit inflammation response and oxidative stress through the activation of ApoE and Nrf2/HO-1 pathway.

SCI consists of two pathological processes that include an immediate primary mechanical injury and a subsequent secondary injury, which exacerbates neurological deficits and outcomes[57]. Numerous studies have indicated that inflammatory response and oxidative stress are two major events of the secondary injury and play contributed roles in complex secondary pathogenesis[6, 58, 59]. During the secondary injury cascade, the infiltrations of inflammatory cells such as macrophages, microglia and neutrophils[60, 61] trigger the release of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6[62–64], leading to cellular necrosis or apoptosis[65]. In addition, inflammatory cells also release excess reactive oxygen species and reactive nitrogen species[66, 67], which causes DNA oxidative damage, proteins oxidation and lipid peroxidation[68]. Necrosis and apoptosis of neurons and glial cells are further induced by this process[69, 70].

EA, a widely used therapy in China, has a good curative effect on SCI and its sequelae[71, 72]. Several studies have shown that EA improves the microenvironment at the epicenter of the spinal cord via reducing edema, inflammatory response, lipid peroxidation and excitatory amino acid toxicity, etc., thus promoting neuronal survival and axonal regeneration and remyelination[73, 74]. In the present study, the data showed that EA significantly accelerated the recovery of neurological function after SCI compared to the control group in WT mice. The histological outcomes were consistent with the functional improvement, which indicates that EA is important in neurologic recovery after SCI. Although several mechanisms mediate the effect of EA on SCI[13–17], the inhibition of inflammatory response and oxidative stress by EA is primary and important factors[20–22]

It is well known that ApoE contributes to modulating neuronal repair and remodeling in CNS trauma and diseases[75, 76], which owes to its anti-inflammatory, anti-oxidative stress and anti-apoptotic properties[77–79]. After SCI, ApoE is found in neutrophils and macrophages at the injury site in the early stages of injury, and in astrocytes during the remodeling of white matter tracts, most prominently in the degenerating parts of the fasciculus gracilis. Meanwhile, the expression of ApoE increases as early as 4 days and reaches to a maximal level between 1 and 2 weeks, and remains up-regulated beyond 3 weeks[80]. In addition, the deficiency of ApoE aggravates inflammatory response and oxidative stress, as well as neural apoptosis, thus retarding the recovery of locomotor and neurological functions from SCI[36].

To analyze the role of ApoE in EA inhibiting inflammation of injured spinal cord, exogenous ApoE-mimetic peptides (i.e., COG112) was used in ApoE deficiency mice which were induced spinal cord injury. COG112 has emerged as a potential candidate for the improvement of neurological and histological outcome following SCI[38]. Supplement of COG112 inhibits the inflammatory response by inhibiting NF- κ B activation[81], diminishes the infiltration of the periphery inflammatory cells into the spinal cord and attenuates demyelination[82], which is consistent with our experimental results. In the present study, we observed that EA can make the mice harbor higher BMS scores with the supplement of COG112 compared to the ApoE-deficiency mice after SCI. The histomorphology and the ultrastructure outcomes were consistent with the functional recovery, including reducing the infiltration of inflammatory cells into the spinal cord, and attenuating axonal demyelination by EA treatment combined with COG112. Conversely, the effect of EA was weakened without the presence of ApoE. It strongly suggests that ApoE is a key target of EA and the effects of EA on functional and morphological recovery depend on the expression of ApoE after SCI. In addition, several studies have shown that inflammatory cytokines, including TNF- α , IL-6, and IL-1 β secreted by activated microglia and macrophages induce cellular death and hinder recovery of neurological function[6, 83]. Previous studies have also shown the ApoE-deficiency significantly increased the release of proinflammatory cytokines(IL-6, and IL-1 β) after SCI stimulation[84]. We show that EA not only reduced the infiltration of the pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β), but also promoted the secretion of anti-inflammatory cytokines (IL-10 and TGF- β 1) combined with COG112 treatment in both WT-control and KO-control groups. These findings support that EA can inhibit inflammatory response by activation of ApoE, and then the latter reduces pro-inflammatory cytokines and increases anti-inflammatory cytokines.

Also, as mentioned above, the Nrf2/HO-1 signaling pathway plays an outstanding role in anti-oxidative activities after SCI[85, 86]. Nrf2 was activated by oxidative stress after injury combined with antioxidant response elements[87] and increased its downstream anti-inflammatory and antioxidant enzymes, including HO-1 and NQO1[86]. In our study, we observed that Nrf2 was elevated after SCI, and Nrf2, HO-1 and NQO1 were further increased by EA treatment combined with COG112, it could be easily predicted that Nrf2/HO-1 signaling pathway may function in oxidative stress after SCI and is involved with the effect of EA on the recovery of SCI. There are a few studies on the interaction between the ApoE and Nrf2 in SCI and other diseases. Yang, et al found ApoE was significantly increased and reached the highest level at the same time with Nrf2 expression. The possible reason is the elevated-ApoE with the anti-oxidant

properties may enhance the Nrf2 expression with the same property[84]. It is reported that the deficiency of ApoE in mice impairs gastric motility functions via the suppression of the activities of dihydrofolate reductase and antioxidant enzymes, such as Nrf2[88]. The lack of ApoE increases oxidative stress response by reducing the expression of Nrf2 and HO-1 after SCI[36]. In a word, the expression levels of Nrf2 genes are closely related to ApoE genotype[89]. In the study, the expression of ApoE is consistent with the expression of Nrf2 after SCI, it suggested that ApoE and Nrf2 played a synergistic role in anti-oxidative stress and anti-inflammation after SCI. The above statements provide the solid supports for our study. Although studies of associations between ApoE and Nrf2 in CNS have been explored, the signaling pathways which regulate the ApoE and Nrf2 have not reached clear conclusions[90]. It needs to be further investigated.

Conclusion

EA improves motor dysfunction, inflammation and demyelination after SCI, and the mechanism is that EA inhibits the inflammatory response and oxidative stress through activation of ApoE and the Nrf2 / HO-1 signaling pathway, respectively. Our data confirms that EA is a reliable treatment for promoting functional recovery in patients with SCI, meanwhile, ApoE is a key target of EA and the effect of EA on SCI depends on the synergy of ApoE and the Nrf2.

Abbreviations

SCI, spinal cord injury. ApoE, apolipoprotein E. Nrf2, nuclear factor erythroid 2-related factor. EA, electroacupuncture. WT, wide type. HO-1, heme oxygenase-1. NQO1, NAD(P)H-quinone oxidoreductase 1. SPF, specific pathogen free. BMS, basso mouse scale. PBS, phosphate buffered saline. HE, hematoxylin/eosin

IF, immunofluorescence. TEM, transmission electron microscopy

WB, western blot. ECL, enhanced chemiluminescence. qRT-PCR, quantitative reverse transcription polymerase chain reaction

SEM, mean \pm standard error of the mean

Declarations

Ethics approval and consent to participate

The project research conformed the principle of medical ethics and the requirements of the declaration of Helsinki, the research design was scientific basis, no unnecessary danger to the subjects. The selected animal species, grade, quantity and specification were suitable. In the experiment, animals were treated well and given anesthesia and analgesia treatment. After the experiment, animals were euthanized. After death, animals were treated innocently.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author contributions

D.N. performed the whole experiments and molecular studies and drafted the manuscript. Z.H.D and D.P. performed the molecular biology study. S. Q. H. and C. L. T. funded and conceived the study, participated in the study design, and revised the manuscript. D.N. and C. L. T. equally contributed to this work. All authors read and approved the final version of the manuscript.

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Figures

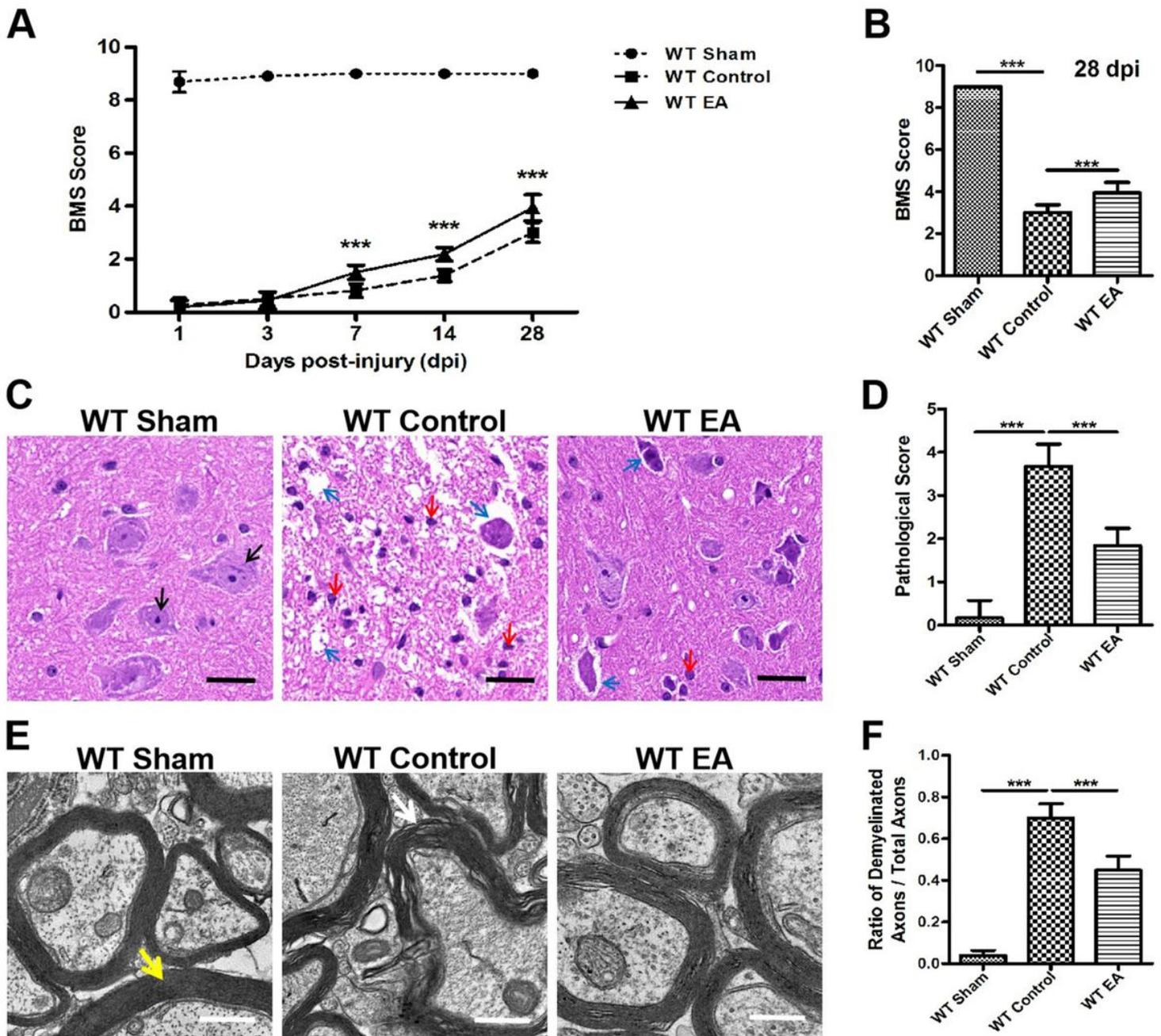


Figure 2

Neurological function assessment and morphological changes after SCI in WT mice (n = 8). A the locomotor function of the hind limb on 1, 3, 7, 14 and 28 dpi were assessed by BMS. B the BMS score on 28 dpi. C the histomorphology of the spinal cord near the epicenter on 28 dpi after SCI by HE staining.

Scale bar = 20 μ m. Black arrows represent normal neurons, while blue arrows represent damaged (apoptotic or necrotic) neurons, and red arrows represent inflammatory cytokines. D Quantitative analysis of pathological score in panel C. E the ultrastructure of the spinal cord on 28 dpi by TEM. Scale bar = 500 nm. Yellow arrow represents myelinated axons, and white arrow represents demyelinated axons. F the ratio of demyelinated axons / total axons in panel E. All data are presented as the mean \pm SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze differences among groups. ***p<0.001.

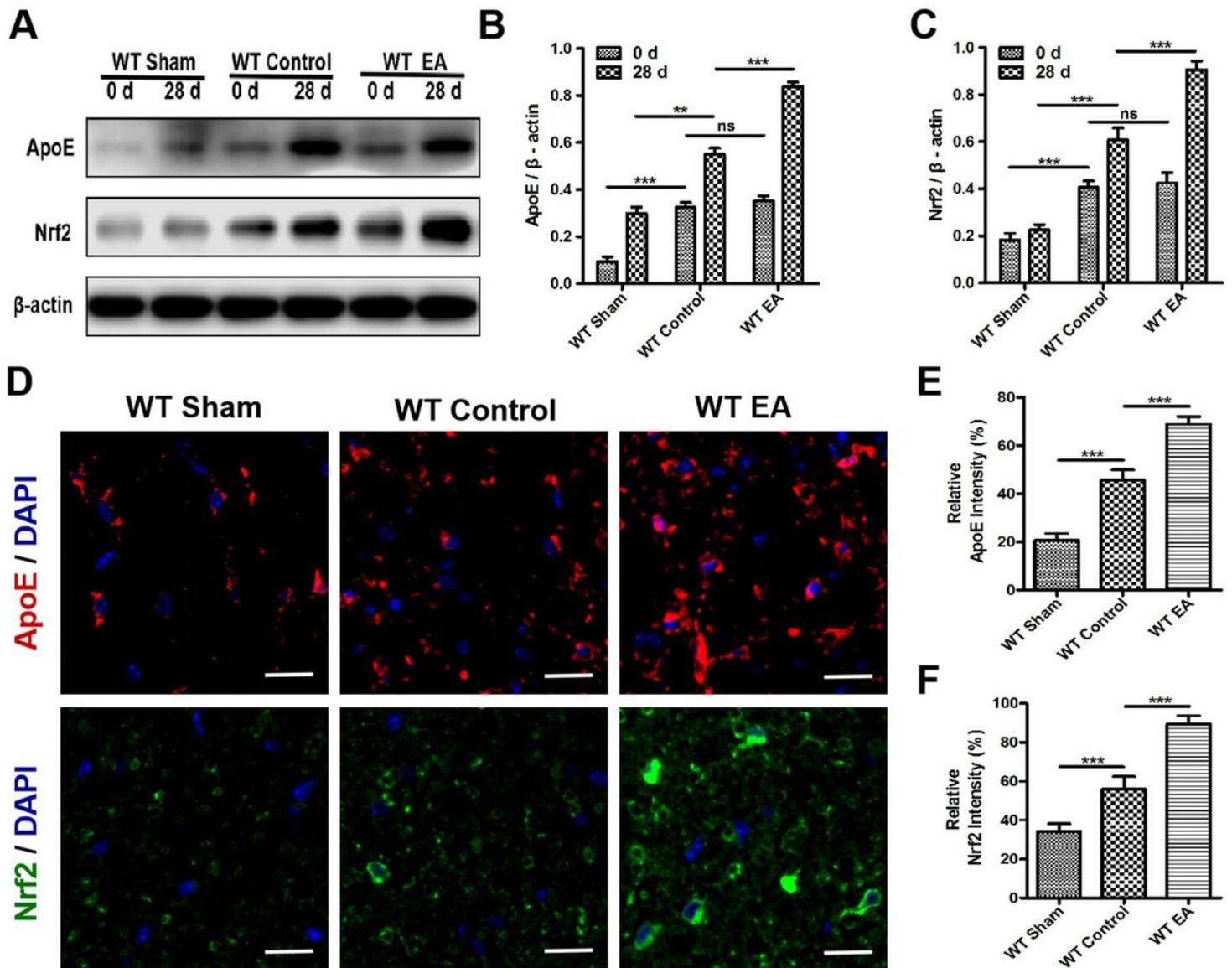


Figure 4

the expression levels of ApoE and Nrf2 on 28 dpi by WB and IF staining (n = 6 per group). A the expression of ApoE and Nrf2 in the spinal cord on 28 dpi by WB. B, C Quantitative analysis of ApoE and Nrf2 protein expression in panel A. D the expressions of ApoE and Nrf2 in the spinal cord on 28 dpi by IF staining. Scale bar = 20 μ m. Red represents ApoE, blue represents cell nucleus, and green represents Nrf2. E, F Quantitative analysis of relative ApoE and Nrf2 fluorescence intensity in panel D. All data are

presented as the mean±SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze differences among groups. ns means no significance, **p<0.01; ***p<0.001.

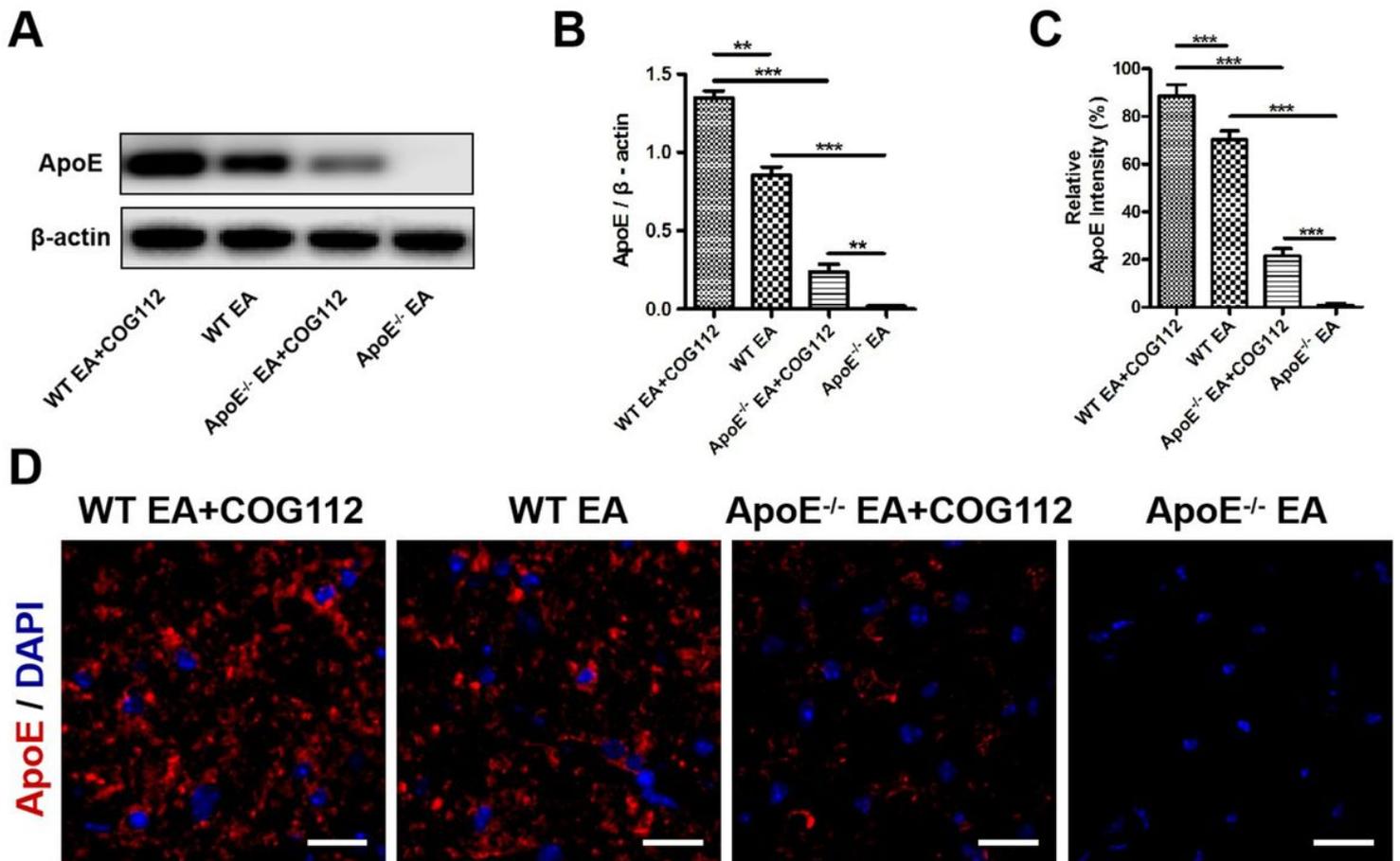


Figure 6

The expression level of ApoE on 28 dpi in the WT and, ApoE^{-/-} mice by WB and IF staining (n = 6 per group). A the expression of ApoE in the spinal cord on 28 dpi by WB. B Quantitative analysis of relative protein expression of ApoE in panel A. C Quantitative analysis of relative ApoE fluorescence intensity in panel D. D the expression of ApoE in the spinal cord on 28 dpi by IF staining. Scale bar = 20μm. Red represents ApoE and blue represents cell nucleus. All data are presented as the mean ±SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze differences among groups. **p<0.01; ***p<0.001.

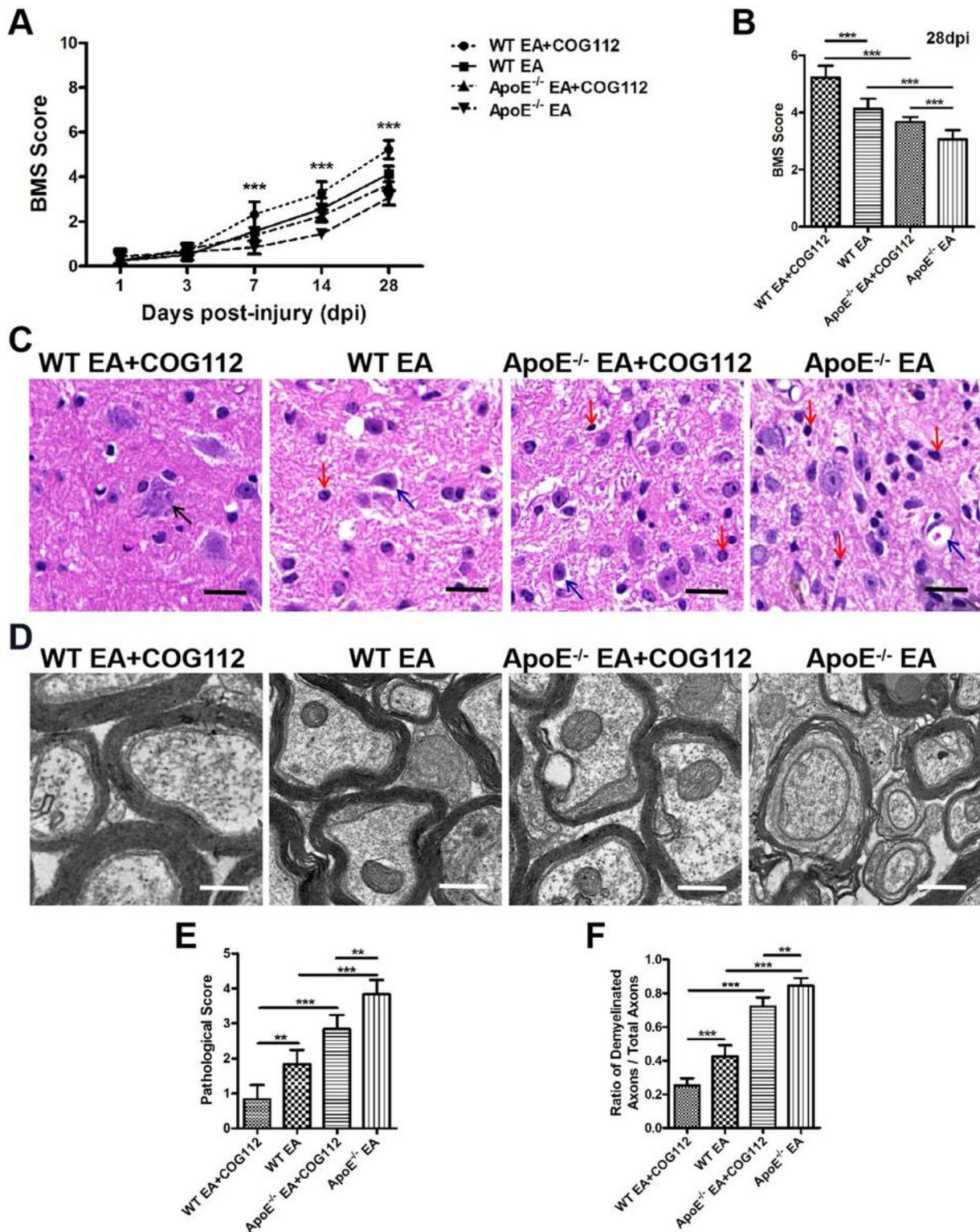


Figure 8

locomotor function and morphology of spinal cord after SCI were assessed by BMS, HE staining and TEM (n = 8 per group). A The locomotor function of hind limb on day 1, 3, 7, 14 and 28 after SCI in the four groups was measured by BMS. B The BMS score on 28 dpi in the four groups. C the histomorphology of spinal cord on 28 dpi in four groups by HE staining. Scale bar = 20 μ m. Black arrows represent normal neurons, while blue arrows represent damaged (apoptotic or necrotic) neurons, and red

arrows represent inflammatory cytokines. D the ultrastructure of spinal cord in the four groups on 28 dpi by TEM. Scale bar = 500 nm. E pathological score in panel C. F the ratio of demyelinated axons / total axons in panel D. All data are presented as the mean±SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyse differences among groups. **p<0.01; ***p<0.001.

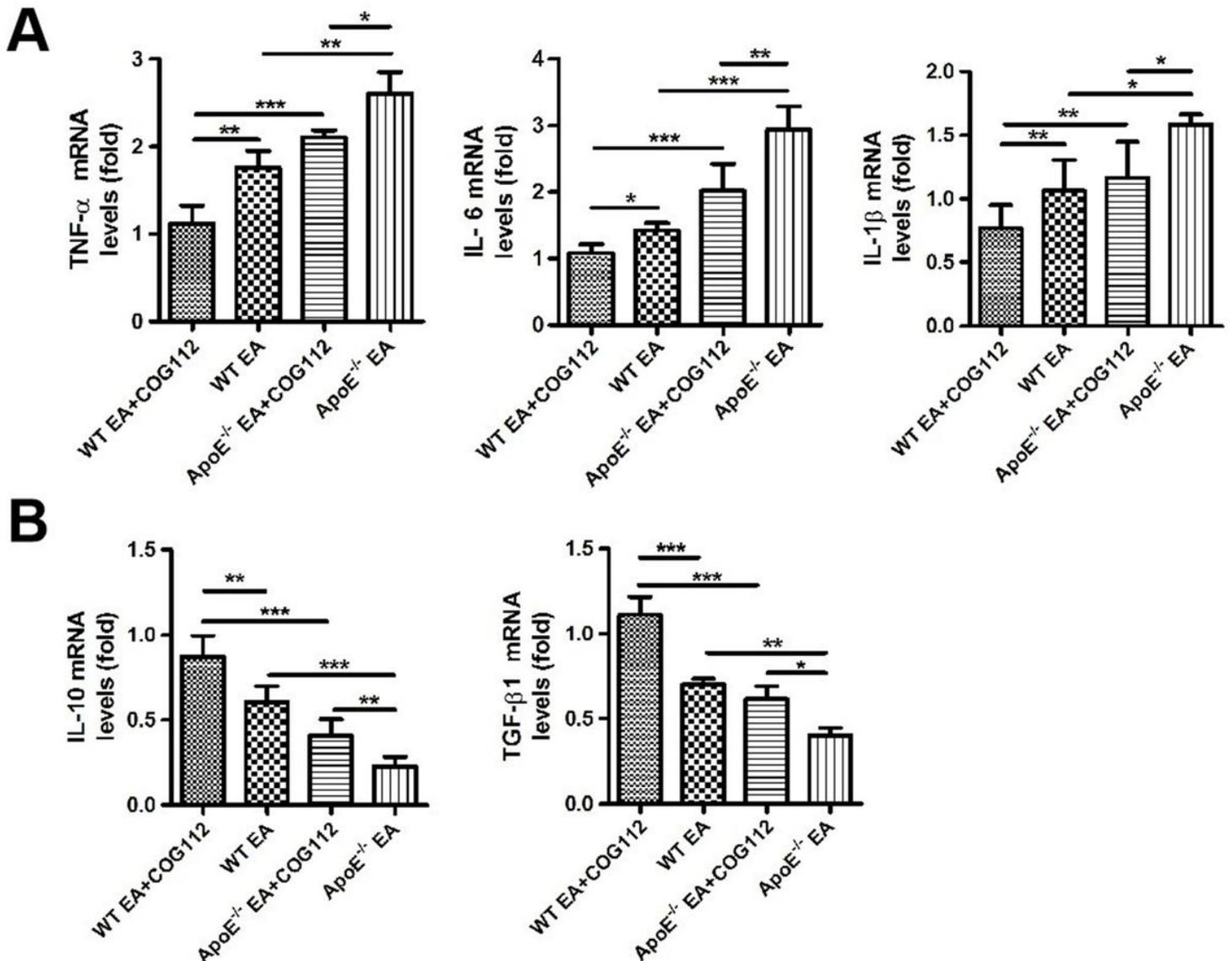


Figure 10

the mRNA levels of inflammatory cytokines on 28 dpi by RT - qPCR (n = 6 per group). A Quantitative analysis of pro-inflammatory cytokines (including TNF-α, IL-6 and IL-1β) mRNA levels. B quantitative analysis of anti-inflammatory cytokines (including IL-10 and TGF-β1) mRNA levels. All data are presented as the mean±SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze differences among groups. *p<0.05; **p<0.01; ***p<0.001.

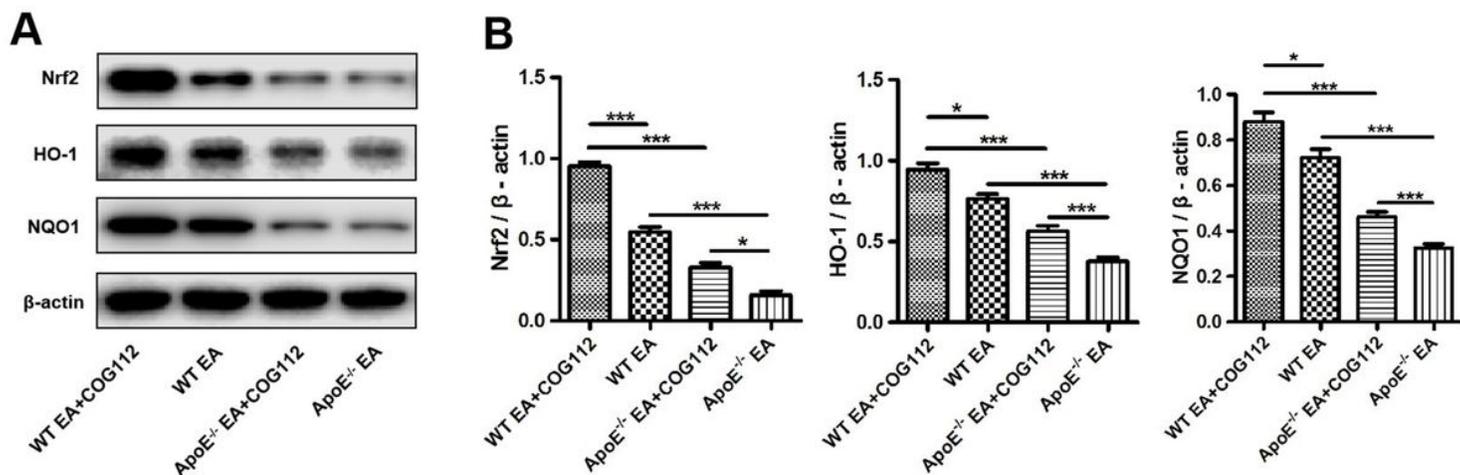


Figure 12

the expressions of Nrf2/HO-1 signaling pathway on 28 dpi by WB (n = 6 per group). A the expressions of Nrf2, HO-1 and NQO1 in the spinal cord on 28 dpi by WB in the four groups. B Quantitative analysis of Nrf2, HO-1 and NQO1 protein expression in panel A. All data are presented as the mean \pm SD. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze differences among groups. * $p < 0.05$; *** $p < 0.001$.