

# Tocotrienol alleviates inflammation and oxidative stress in a rat model of spinal cord injury via suppression of transforming growth factor- $\beta$

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**Abstract.** In recent years accumulating evidence has indicated that tocotrienol exhibits an oxidation resistance function, decreased cholesterol function, inhibits cancer function and has unique physiological functions, including anti-inflammatory, anti-apoptotic and anti-oxidative properties. The present study investigated the effect of tocotrienols on spinal cord injury (SCI) by evaluating oxidative stress, inflammation and inducible nitric oxide synthase (iNOS) in rats. A rat model of SCI was induced by operation. SCI rats were treated with 120 mg/kg/day tocotrienol once a day for eight consecutive weeks. Functional recovery following SCI was measured by using the Basso Beattie Bresnahan (BBB) locomotor rating scale. Then the volume of spinal cord contusions was measured following induction of SCI in the rats. In SCI rats, serum malondialdehyde, superoxide dismutase, catalase, glutathione peroxidase, nuclear factor- $\kappa$ B p65 unit, tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 levels were analyzed using respective commercial immunoassay kits. Firstly, iNOS, transforming growth factor (TGF)- $\beta$ , collagen type IV and fibronectin protein expression levels, in addition to iNOS activity and plasma nitric oxide (NO) production in SCI rats was analyzed using western blot analysis, commercial kits and Griess reagent, respectively. Tocotrienol treatment elevated BBB scores and contused volume in the SCI rats. Tocotrienol protected against SCI with reduced oxidative stress and inflammation, and inhibited iNOS protein

expression iNOS activity and plasma NO production in rats. In addition, treatment with tocotrienols suppressed TGF- $\beta$ , collagen type IV and fibronectin protein expression levels in SCI rats. These results suggest that tocotrienols protect SCI, and suppress oxidative stress, inflammation and iNOS in this model of SCI through TGF- $\beta$ , collagen type IV and fibronectin signaling pathways.

## Introduction

Spinal cord injury (SCI) is a common type of motor system trauma which may cause varying degrees of four limbs or paraplegia with loss of labor ability, potentially resulting in a heavier burden on society and families in 2010 (1). Epidemiological data indicated that in the USA, traffic accidents and high-altitude falls were the predominant causes of SCI, accounting for ~67.5%. The majority of cases were in young men with an average age of 41.0 years, the annual average morbidity is ~40 per million people, and ~12,000 people per year and increasing (2). At present in the field of sports injury, recovery after SCI remains difficult. However, scholars worldwide continue to investigate new treatment strategies for SCI.

At present the SCI mechanism is divided into primary and secondary SCI (3). The former consists of instantaneous mechanical damage that is reversible. Secondary damage gradually forms in the weeks following primary damage, and is accompanied by a series of cell metabolism and gene expression changes, including excitatory amino acid release, free radical damage, decrease in ion inflow, inflammation and cell apoptosis, resulting in more harm than primary damage (4,5). Numerous studies have shown that post changes of SCI gene expression play an important role in the pathological process (4,5).

Transforming growth factor (TGF)- $\beta$  is a multifunctional cytokine, and its function in mammals involves damage repair and the formation of scar tissue (6). The biological function of TGF- $\beta$  is mediated via the conduction of types I (RI) and II (RII) transmembrane receptors (7). TGF- $\beta$  is present in almost all cells; therefore, it can influence the physiological function of the majority of tissues correspondingly (8). *In vitro*, TGF- $\beta$

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is a potent agent for astrocyte chemotaxis, which can result in the hypertrophy of astrocytes and upregulation of the synthesis of fibronectin and collagen IV (8).

Tocotrienols are isomers of vitamin E, located primarily in plants, with antioxidative (9), anti-tumor (10) and neuro-protective functions (11). Tocotrienols inhibit proliferation of breast cancer cells, show G1 phase retardation and can promote cell apoptosis (12,13). However, to the best of our knowledge, the potential protective effect and underlying molecular mechanism of tocotrienol on SCI has not been investigated. In addition, investigated the hypothesis that the effect of tocotrienol on SCI is mediated via the suppression of TGF- $\beta$ .

### Materials and methods

**Animals and surgery.** A total of 45 female Sprague Dawley rats weighing 250-270 g were obtained from the Animal Resource Center of Xinjiang Medical University (Urumqi, China). Animal experiments were performed in accordance to the institutional guidelines provided by the Committee on Animal Research at Xinjiang Medical University.

**Drugs and chemicals.** Tocotrienol [D-(-)-Tocotrienol; purity, >98%] was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), shown in Fig. 1. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), activity of glutathione peroxidase (GSH-PX), NF- $\kappa$ B p65 unit, tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6 ELISA kits were purchased from R&D Systems, Inc. (catalog nos. A003-1, A001-3, A007-2, A005, H202, H052, H002 and H007; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A bicinchoninic assay kit was bought from Beyotime Institute of Biotechnology (Nantong, China). Inducible nitric oxide synthase (iNOS) commercial kit was bought from Imgenex (catalog no. A014-1; Nanjing Jiancheng Bioengineering Institute).

**Experimental groups and procedures.** Sprague Dawley rats were randomly divided into four groups (n=10 per group): i) Sham group, normal rats received physiological saline 0.1 ml/100 g (i.p.); ii) SCI group (SCI) (14), SCI model rats received physiological saline 0.1 ml/100 g (i.p.); iii) methylprednisolone sodium succinate (MPSS) group (positive control group), SCI model rats were treated with 100 mg/kg MPSS (i.p.); and iv) tocotrienol group, which experienced SCI and received 120 mg/kg/day tocotrienol once a day for eight consecutive weeks (15).

**Evaluation of Basso Beattie Bresnahan (BBB) locomotor rating scale.** The evaluation of locomotor function after treatment with tocotrienol on SCI was performed using the BBB locomotor rating scale of 0 (complete paralysis) to 21 (normal locomotion) (16).

**Evaluation of the volume of spinal cord contusions after SCI.** The volume of spinal cord contusions after SCI was evaluated by calculating as wet weight after treatment with doctriinal on SCI. Spinal cord tissue samples were dried at 80°C for 48 h and calculated as dry weight. The volume of spinal cord

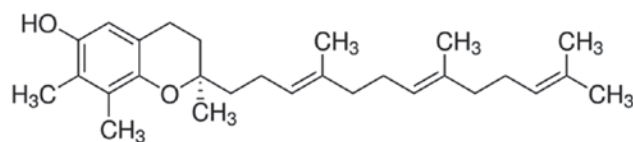


Figure 1. Chemical structure of tocotrienol.

contusions was calculated by the following formula: Water content of spinal cord (%)=(Wet weight-dry weight)/wet weight x 100%.

**Evaluation of serum oxidative stress and inflammation.** After the experimental administration of tocotrienol on SCI rats, serum was collected for analysis. Levels of MDA, SOD, CAT, GSH-PX, NF- $\kappa$ B p65 unit, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were analyzed using respective commercial immunoassay kits according to the manufacturers protocols (Wuhan Elabscience Biotechnology, Co., Ltd., Wuhan, China).

**Western blot analysis.** Spinal cord tissue samples (50 mg) were homogenized in an ice-cold lysis buffer (Beyotime Institute of Biotechnology). The mixed liquor was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was analyzed protein quantification using a bicinchoninic acid assay kit (catalog no. P0013B; Beyotime Institute of Biotechnology). Total proteins (10  $\mu$ g) were separated by electrophoresis using 10-12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Merck Millipore). Membranes were washed with Tris buffer saline Tween-20 (TBST) three times and blocked with 5% skim milk powder in TBST for 1 h at 37°C. Subsequently, they were incubated using anti-iNOS (catalog no. sc-8310; 1:2,000), anti-TGF- $\beta$  (catalog no. sc-130348; 1:2,000), anti-collagen type IV (catalog no. sc-9301; 1:3,000), anti-fibronectin (catalog no. sc-69681; 1:4,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) anti- $\beta$ -actin (catalog no. D110007; 1:2,000; Sangon Biotech, Shanghai, China) primary antibodies at 4°C overnight. Then, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h and visualized using enhanced chemiluminescence agents (ECL Plus; Pierce Protein Biology; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The optical densities of immunopositive bands were determined using Gene Tools analysis software (GeneTools, LLC, Philomath, OR, USA).

**Determination of iNOS activity.** Following experimentation, spinal cord tissues were collected from different groups. iNOS activity was determined using ELISA commercial kits according to the manufacturer instructions (catalog no. A014-1; Nanjing Jiancheng Bioengineering Institute).

**Evaluation of plasma NO production.** Following experimentation, the plasma supernatant was collected and underwent 12,000 x g centrifugation for 20 min at 4°C. Nitrite concentration was determined using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid (Sangon Biotech) at a wavelength of 450 nm

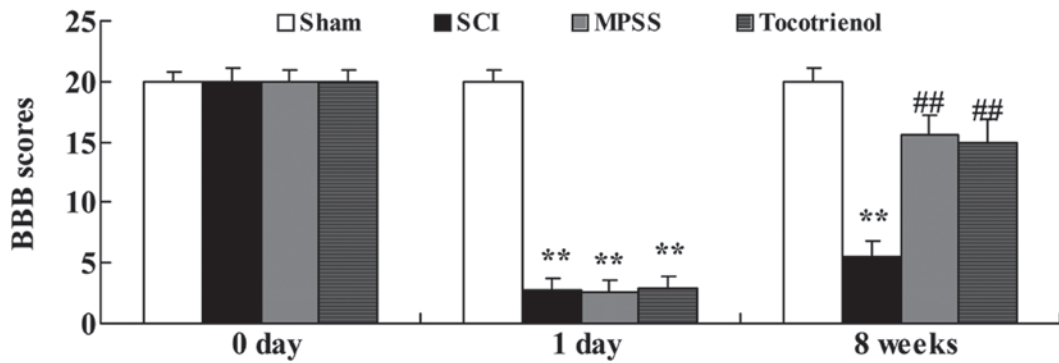


Figure 2. Effects of tocotrienol on functional recovery after SCI <sup>\*\*</sup>P<0.05 vs. sham group; <sup>##</sup>P<0.05 vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg)-treated.

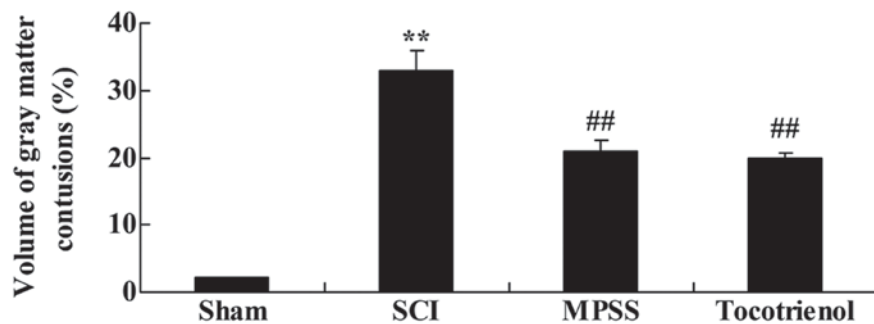


Figure 3. Effects of tocotrienol on the volume of gray matter contusions in the injured spinal cords. <sup>\*\*</sup>P<0.05 vs. sham group; <sup>##</sup>P<0.01 vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg)-treated.

by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation and analyzed using one-way analysis of variance followed by Dunnett's test. All statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of tocotrienol on functional recovery after SCI.** To assess the effects of tocotrienol on functional recovery after SCI, functional recovery was assessed using BBB behavioral testing one day before SCI, one day post-SCI, and for eight weeks post-SCI. Reduced BBB scores were observed in SCI group at one day post-SCI or eight weeks post-SCI, compared to that of control group (P<0.05, Fig. 2). At one day post-SCI, BBB scores were not significantly different in the MPSS or tocotrienol groups compared with the SCI group (Fig. 2). However, at eight weeks post-SCI, BBB scores were significantly higher in MPSS and tocotrienol groups compared with the SCI group (P<0.05, Fig. 2). There was no significant difference between MPSS and tocotrienol groups at any time point (P>0.05, Fig. 2).

**Effect of tocotrienol on the volume of gray matter contusions in the injured spinal cords.** Next, the effects of tocotrienol

were investigated on the volume of gray matter contusions in the injured spinal cords. Contused volume was significantly increased in the SCI group, compared with the control group (P<0.05, Fig. 3). Furthermore, contused volume was significantly reduced in the tocotrienol and MPSS groups compared with the SCI group (P<0.05, Fig. 3). However, there was no significant difference between the MPSS and tocotrienol groups (P>0.05, Fig. 3).

**Effect of tocotrienol on oxidative stress.** To elucidate the effects of tocotrienol on oxidative stress in SCI rats, the serum levels of MDA, SOD, CAT and GSH-PX were evaluated. Initially, serum MDA levels were elevated and serum SOD, CAT and GSH-PX levels were suppressed in the SCI group compared with the control group (P<0.05, Fig. 4). Furthermore, these SCI-induced changes in serum MDA, SOD, CAT and GSH-PX levels were significantly reversed by the MPSS and tocotrienol treatments, compared with the SCI group (P<0.05, Fig. 4). However, no significant inter-group difference was observed between the MPSS and tocotrienol groups for anti-oxidative effects in SCI model rats (P>0.05, Fig. 4).

**Effect of tocotrienol on inflammation.** To elucidate the effects of tocotrienols on inflammation of SCI rat, the serum levels of NF- $\kappa$ B p65 unit, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were evaluated. SCI induced inflammation reactions in rats, compared to the control group, as indicated by significantly elevated levels of the detected proteins (P<0.05, Fig. 5). Notably, the serum NF- $\kappa$ B p65 unit, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels were significantly

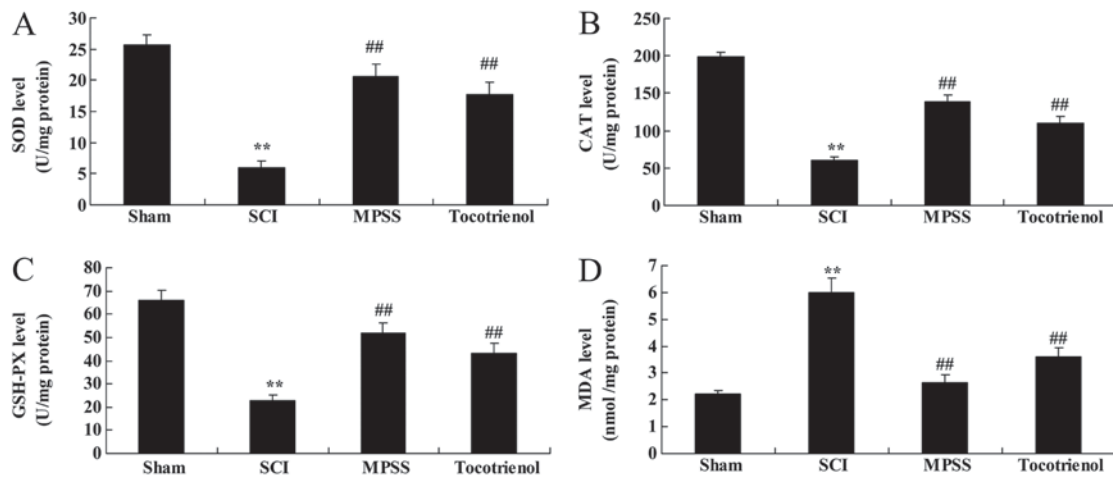


Figure 4. Effects of tocotrienol on oxidative stress. The effects of tocotrienol on (A) SOD, (B) CAT, (C) GSH-PX and (D) MDA levels in SCI rats. \*\* $P < 0.05$  vs. sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg) -treated; SOD, superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde.

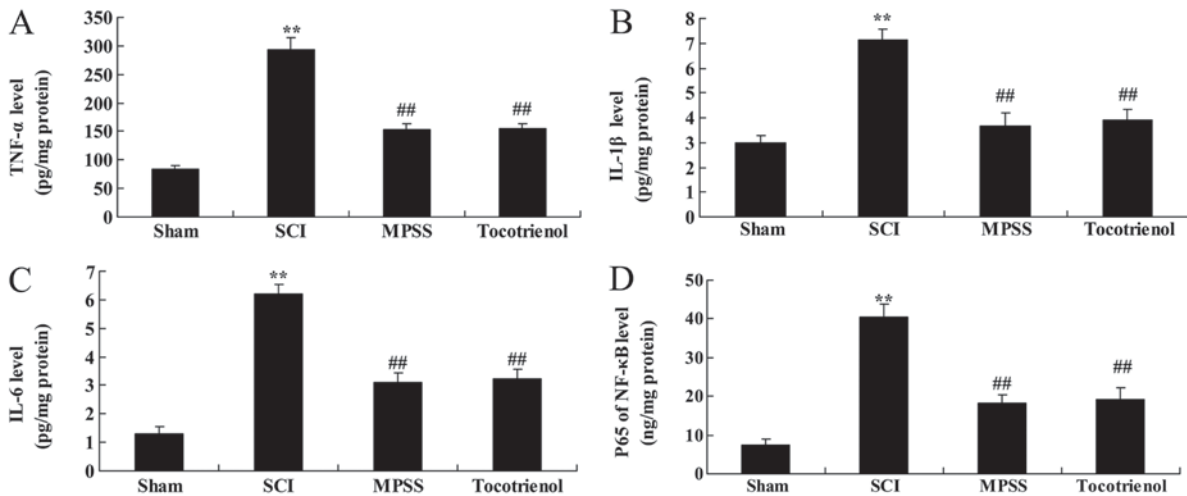


Figure 5. Effects of tocotrienol on inflammation. The effects of tocotrienol on (A) TNF- $\alpha$ , (B) IL-1 $\beta$ , (C) IL-6 and (D) NF- $\kappa$ B p65 unit in SCI rats. \*\* $P < 0.05$  vs. sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg) -treated; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-6, interleukin-6; NF- $\kappa$ B p65 unit, nuclear factor- $\kappa$ B p65 unit.

reduced by treatment with MPSS and tocotrienol, compared to the SCI group ( $P < 0.05$ , Fig. 5). However, no significant changes in the serum levels of NF- $\kappa$ B p65 unit, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were detected between the MPSS and tocotrienol groups ( $P > 0.05$ , Fig. 5A-D).

**Effect of tocotrienol on iNOS expression, iNOS activity and plasma NO production.** To explain the effects of tocotrienol on iNOS protein expression in SCI rats, iNOS protein expression, iNOS activity and plasma NO production were evaluated. SCI promoted iNOS protein expression in rats compared with the control group ( $P < 0.05$ , Fig. 6A and B). Treatment with MPSS and tocotrienol reduced iNOS protein expression compared to the SCI group ( $P < 0.05$ , Fig. 6A and B). SCI activated iNOS activity and increased plasma NO production in SCI rats compared with the control group ( $P < 0.05$ , Fig. 6C and D). However, these changes were significantly reversed by treatment with MPSS and tocotrienol compared to the SCI group ( $P < 0.05$ , Fig. 6C and D). Furthermore, no significant

differences were detected in iNOS protein expression, iNOS activity and increased plasma NO production between the MPSS and tocotrienol groups ( $P > 0.05$ , Fig. 6A-D).

**Effect of tocotrienol on the expression of TGF- $\beta$ .** Western blot analysis was used to further elucidate the effects of tocotrienol on the expression of TGF- $\beta$  protein in SCI rats. Western blot analysis demonstrated that the expression of TGF- $\beta$  protein was significantly elevated in the SCI group compared with the sham group ( $P < 0.05$ , Fig. 7A and B). By contrast, treatment with MPSS and tocotrienol significantly inhibited TGF- $\beta$  protein expression, compared with the SCI group ( $P < 0.05$ , Fig. 7A and B). However, there was no significant difference between the MPSS and tocotrienol groups ( $P > 0.05$ , Fig. 7A and B).

**Effect of tocotrienol on the expression of collagen type IV.** Western blot analysis was used to evaluate the effects of tocotrienol on the protein expression levels of collagen type IV

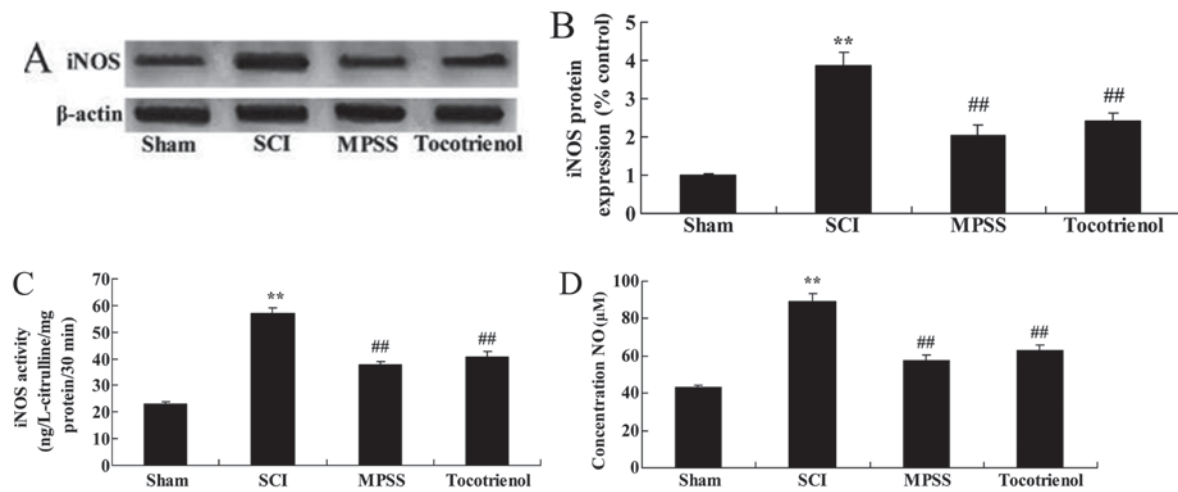


Figure 6. Effects of tocotrienol on iNOS expression, iNOS activity and plasma NO production. The effects of tocotrienol on iNOS protein expression by (A) western blot assays, and statistical analysis of (B) iNOS protein expression levels, (C) iNOS activity and (D) plasma NO production in SCI rats. \*\* $P < 0.05$  vs. sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol tocotrienol (120 mg/kg)-treated; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

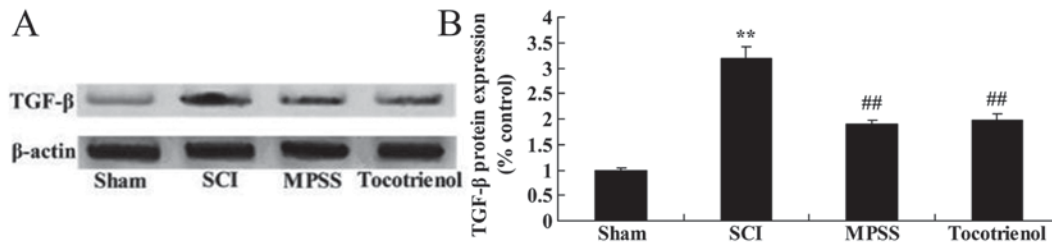


Figure 7. Effects of tocotrienol on the expression of TGF- $\beta$ . The effects of tocotrienol on TGF- $\beta$  protein expression as showed by (A) western blot assay and (B) statistical analysis of TGF- $\beta$  protein expression levels in SCI rats. \*\* $P < 0.05$  vs. sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg)-treated; TGF- $\beta$ , transforming growth factor- $\beta$ .

in SCI rats. The expression of collagen type IV protein was significantly increased in SCI model group, compared to the sham group ( $P < 0.05$ , Fig. 8A and B). However, MPSS and tocotrienol significantly reduced the collagen type IV protein expression compared with the SCI group ( $P < 0.05$ , Fig. 8A and B). However, there was no significant difference in collagen type IV protein expression between the MPSS and tocotrienol groups ( $P > 0.05$ , Fig. 8A and B).

**Effect of tocotrienol on the expression of fibronectin.** Western blot analysis was used to further evaluate the effects of tocotrienol on the expression of fibronectin protein in SCI rats. As shown in Fig. 9A and B, there was a significant increase in fibronectin protein expression in the SCI model rats compared with the sham group. In contrast to the SCI model rats, MPSS and tocotrienol treatments significantly suppressed the protein expression of fibronectin ( $P < 0.05$ , Fig. 9A and B). However, in the expression no significant changes in fibronectin protein expression were detected between the MPSS and tocotrienol groups were observed ( $P > 0.05$ , Fig. 9A and B).

## Discussion

As a result of the development of modern transportation and industrial and mining processes, SCI has become a commonly reported condition in clinical practice (14). The two damage

mechanisms of primary and secondary SCI may combine to seriously affect human health and quality of life; potentially causing disabling injuries (14). Primary SCI occurs instantly after injury of spinal cord tissue due to exposure to external forces, and is an irreversible form of damage (17). On the basis of primary injury in the spinal cord tissue, the progressive, self-destructive cascade amplification destruction process caused by a variety of factors is known as secondary SCI; the degree of damage is substantially more than in primary SCI (17-19). The present study further showed that BBB scores and contused volume in rats post-SCI were improved and suppressed by treatment with tocotrienol, respectively. Frank *et al* (18) reported that tocotrienol has potential as a neuroprotective dietary factor.

Mitochondrial dysfunction is an important factor that causes the deaths of a large number of nerve cells following SCI; this occurrence is directly associated with the accumulation of  $Ca^{2+}$  in the cell after the damage (20). Post-SCI oxidative stress damages the ion steady state inside and outside the mitochondrial membrane,  $Ca^{2+}$  accumulates inside the mitochondria, causing mitochondrial damage and aerobic energy metabolic disorders and inhibiting the synthesis of ATP (4). Following SCI, excessive activity of free radicals may be applied to the postsynaptic neuron, activating nearby astrocytes and microglia, causing ionic imbalance at the neuronal cell membrane (21). In addition,  $Ca^{2+}$ -ATPase of the plasma

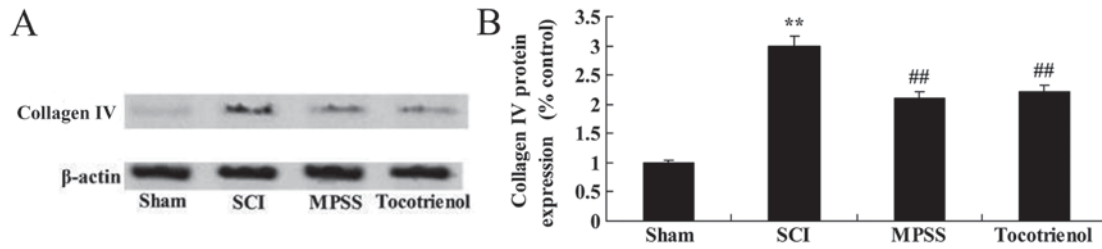


Figure 8. Effects of tocotrienol on the expression of collagen type IV. The effects of tocotrienol on collagen type IV protein expression by (A) western blot assay and (B) statistical analysis of collagen type IV protein expression levels in SCI rats. \*\* $P < 0.05$  vs. sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg)-treated.

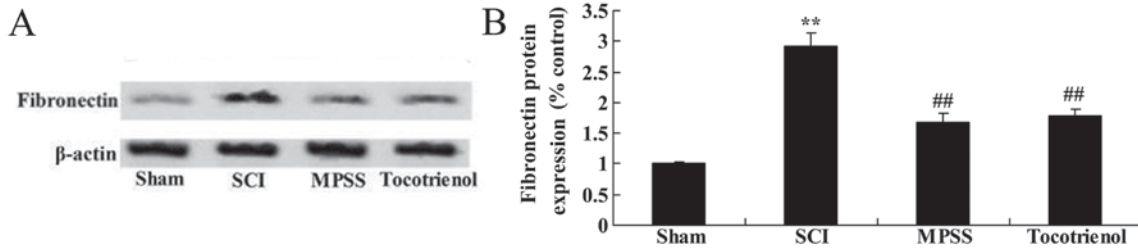


Figure 9. Effects of tocotrienol on the expression of fibronectin. The effects of tocotrienol on fibronectin protein expression by (A) western blot assay and (B) statistical analysis of fibronectin protein expression levels in SCI rats. \*\* $P < 0.05$  vs. Sham group; ## $P < 0.05$  vs. SCI group. Sham, Sham group; SCI, spinal cord injury group; MPSS, methylprednisolone-treated; Tocotrienol, tocotrienol (120 mg/kg)-treated.

and endoplasmic reticulum on the retina is highly sensitive to oxidative damage. Following SCI, the lipid peroxidation process can inhibit the activity of  $\text{Ca}^{2+}$ -ATPase and interfere with the movement of  $\text{Ca}^{2+}$  out of the cell membrane, causing intracellular  $\text{Ca}^{2+}$  overload and ionic imbalance (4). The present results suggest that the neuroprotective effect of tocotrienol reduced the serum MDA level and increased the serum SOD, CAT and GSH-PX levels in SCI rats (21). Siddiqui *et al* showed that protective effects of tocotrienol may protect against nephropathy in experimental type-2 diabetic rats by suppression of oxidative stress (21). Nizar *et al* (22) found that the antioxidative effect of tocotrienol protects osteoblasts through reduction of oxidative stress.

Inflammation serves a crucial function in the onset of SCI, inflammatory reaction of local damage tissue can increase the degree of secondary SCI (23). Following local damage, the secondary inflammatory environment may cause the injury of spinal cord cell necrosis and apoptosis, and irreversible pathological changes, thus aggravating SCI (24). Neutrophils may gather to the site of injury and release materials such as elastase; aggravating tissue edema, necrosis and promoting apoptosis of neurons and oligodendrocytes, and leading to local glial scar (24). The present findings support the protective effects of tocotrienol, which inhibited the serum levels of NF- $\kappa$ B p65 unit, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in SCI rats. González *et al* (25) indicated that tocotrienols exhibit anti-inflammatory properties and protect from arginine chronic-like pancreatitis. Yam *et al* (26) suggested that tocotrienols suppress proinflammatory markers in RAW264.7 macrophages.

Numerous studies have shown that central nerve damage caused by ischemia and trauma can lead to substantial and persistent expression of neuronal iNOS, and synthesis of excessive quantities of NO, which has direct neurotoxic

effects (27). NO is a type of small molecular free radical with a wide range of biological activity, including vasodilation, nerve information transfer and cytotoxic effects (27). NO and iNOS are crucially involved in central nervous system injury. Excessive NO can induce cell apoptosis, and apoptosis is believed to be important factor of SCI secondary change (28). The present results showed that the protective effects of tocotrienol reduced iNOS protein expression, iNOS activity and plasma NO production in SCI rats. Qureshi *et al* reported that tocotrienol inhibits lipopolysaccharide-induced proinflammatory cytokines through suppression of iNOS gene expression in macrophages of female mice (29).

The positive expression of TGF- $\beta$  is visualized as red granular staining in the cytoplasm of articular cartilage in rats (30). The expression level of TGF- $\beta$  in rats with cartilage articular is relatively low except that the expression on the surface of the articular cartilage is significantly higher than that of the middle and deep layer of cartilage (31). It has been previously reported that TGF- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 are strongly expressed on the surface layer, transitional layer and the low-maturity cell layer of cartilage cells during the process of chondrogenesis (30). Experiments have confirmed that TGF- $\beta$  is expressed in the early stage of cartilage formation, in cartilage callus mesenchymal cells, immature chondrocytes and mature chondrocytes (32). Furthermore, previous studies show that the extracellular expression of TGF- $\beta$ 1 initially appeared in the hematoma in injured area after the spinal cord injury, and the expression is subsequently enhanced in the cytoplasm and nucleus of astrocytes, intramedullary and extramedullary capillary endothelial cells and motor neurons (33,34). The present results indicate that treatment with tocotrienols significantly inhibited TGF- $\beta$  protein expression in SCI rats. Siddiqui *et al* (21) reported that tocotrienol mitigates the damage of lipid-induced nephropathy through suppression of TGF- $\beta$  and collagen type IV.

Fibronectin is a multifunctional glycoprotein found in the extracellular matrix and plasma (35). Widely existing in animal tissues and tissue fluid, fibronectin is a V-shaped macromolecular glycoprotein with a molecular weight of ~450 kDa and a variety of biological activities (36). Numerous international studies have shown that the fibronectin molecule is highly conserved in the evolution process, and various animal body fluids share similar structure, properties and biological function, therefore, fibronectin from different organisms may be interchangeable (33,34,37). Fibronectin has many different subunits which are the expression products of the same gene but different in the RNA splicing after transcription and thus different mRNA are produced (38). Fibronectin is widely distributed in hematoma, the surface of numerous types of cells and extracellular matrix (36). Fibronectin is crucially involved in cell adhesion, migration, differentiation and functions. In recent years, fibronectin has been found to play an important role in the occurrence and development of arthritis and cartilage degeneration (39). In the present study, tocotrienol treatment significantly reduced the collagen type IV and fibronectin protein expression levels of SCI rats. González *et al* reported that tocotrienol prevents against lipid-induced nephropathy through suppression of TGF- $\beta$  and collagen type IV (25).

SCI can directly cause necrosis and apoptosis of vascular endothelial cells, basement membrane components and structural damage of the microvascular wall, which are contributed to the disruption of the blood-spinal cord barrier and lead to the leakage of vascular material, and further aggravate the inflammatory reaction, edema and other secondary injury (40). As the basement membrane component, collagen IV and laminin begin degradation following spinal cord injury; however, the secretion of collagen IV and laminin are enhanced accordingly as the mechanism of vascular regeneration is activated rapidly by pathophysiological requirements (41). Previous results have shown that the early formation of collagen IV and laminin after spinal cord injury contribute to the restriction of inflammatory reaction and the promotion of neovascularization (40,41). Furthermore, pretreatment with tocotrienol treatment significantly reduced the protein expression of fibronectin in SCI rats, in the present study. González *et al* (25) reported that oral tocotrienols improve quantitative measures of chronic pancreatic damage through fibronectin.

In conclusion, tocotrienol attenuated the functional recovery and the volume of gray matter contusions of SCI rats. Prevention of oxidative damage, inflammation and iNOS may be the key mechanisms underlying the effect of tocotrienol on SCI through collagen type IV and fibronectin, thereby promoting functional recovery in SCI rats, potentially in part via the TGF- $\beta$  signaling pathway.

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