Original Article

The Effect of 2,5-hexanedione on Myelin Protein Zero Expression, and Its Mitigation Using Ginkgo Biloba Extract*

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Abstract

Objective To investigate the role of myelin protein zero (P_0) in 2,5-hexanedione (2,5-HD)-induced peripheral nerve injury, and the protective effect of Ginkgo biloba extract (Egb761) on 2,5-HD-induced toxic peripheral neuropathy.

Methods After 4 weeks of treatment with 2,5-HD at different doses (50, 100, 200, 400 mg/kg) in rats, changes in the levels of P_0 in rat sciatic nerves was investigated, and the effect of Egb761 on 2,5-HD-induced toxic peripheral neuropathy was studied.

Results The blood-nerve barrier (BNB) permeability of the sciatic nerve increased, and the expression of P_0 mRNA and P_0 protein decreased in a dose-dependent manner after treatment with 2,5-HD for 4 weeks. Pretreatment with Egb761 protected against BNB interruption, and inhibited P_0 mRNA and protein reduction during 2,5-HD treatment. Pretreatment with Egb761 significantly reduced loss of body weight (P<0.01) and mitigated gait abnormalities (2.85±0.22) induced by 400 mg/kg 2,5-HD (P<0.01). It also reduced the signs of neurotoxicity induced by 2,5-HD.

Conclusion 2,5-HD inhibited the expression of P_0 in a dose-dependent manner, and this may be an important mechanism by which toxic peripheral neuropathy is induced by 2,5-HD. Egb761 has a protective effect against 2,5-HD-induced peripheral neurotoxicity in rats.

Key words: Myelin protein zero; 2,5-hexanedione; Ginkgo biloba extract

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INTRODUCTION

The aliphatic hydrocarbon solvent n-hexane is widely used in adhesives, polishes, rubber, paint, and ink, as well as in food processing, for example in the extraction of vegetable oil. Chronic exposure to *n*-hexane produces nerve damage characterized as central-peripheral neuropathy[1].

Metabolism studies suggest that 2,5-hexanedione (2,5-HD) is the active metabolite of n-hexane that is responsible for mediating the neurotoxicity of the parent compound[2]. Neuropathology examination revealed a characteristic pattern of scattered, focal paranodal and internodal axonal swelling, corresponding

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thinning of myelin, segmental demyelination and distal axonal Wallerian-type degeneration in peripheral nerves.

In the peripheral nerve system (PNS), the myelin sheath is a coating composed of many layers of specialized cell membranes generated using Schwann cells. It surrounds most large axons of peripheral neurons and aids high fidelity signal transduction and rapid signal conductance[3]. Myelin protein zero (P_0) is the major adhesive and structural element of peripheral myelin. It mediates the attachment of Schwann cell membranes as they encase axons to generate compact myelin. P_0 can also promote the regeneration of injured axons[4]. Therefore, changes in P_0 can influence the integrated structure and function of myelin. However, it is not clear if P_0 expression is altered during toxic peripheral neuropathy induced by 2,5-HD.

The dried leaves of the Ginkgo tree can be extracted to give a Ginkgo biloba extract (Egb761). This extract is composed of 24% ginkgo flavone glycosides and 6% terpene lactones. Its biological activities include anti-inflammatory activity[5], free radical scavenging, platelet-activating factor antagonization, endothelium derived relaxing factor stimulation, suppression of peripheral benzodiazepine receptor expression in the cerebral cortex and cellular metabolism modulation, among other activities[6]. Previous reports have demonstrated that Egb761 is beneficial in the treatment of Alzheimer's disease[7], Parkinson's disease[8], coronary heart disease, hypertension, hypercholesterolemia and diabetes mellitus[9]. Animal studies have also shown that it can improve spatial memory and motivation[10], and attenuate neuropathic pain[11]. Egb761 was recently found to promote the regeneration of injured peripheral nerves and to improve peripheral nerve function recovery[12-13]. It has also been found to improve functional measures in individuals with multiple sclerosis[14]. The aim of the present study was to investigate whether Egb761 has a protective effect against toxic peripheral neuropathy induced by 2,5-HD.

MATERIALS AND METHODS

Chemicals

2,5-HD (>97%) was manufactured by Alfa Aesar (Ward Hill, MA, USA), and Evans blue (EB) and formamide were obtained from Sigma-Aldrich (St Louis, MO, USA). Egb761 was manufactured by Dr. Willmar Schwabe GmbH & Co. (Karlsruhe, Germany). Paraformaldehyde and other chemicals were manufactured by the Beijing Chemical Reagents Company (Beijing, China) or Sigma-Aldrich.

Animals and Treatments

Two-month-old male Wistar rats (200-250g, obtained from the Laboratory Animal Breeding Center of Peking Union Medical College, Beijing, China) were housed in the departmental animal facility with controlled temperature and humidity, and with food and water available *ad* libitum. Animals were allowed to adapt to their new surroundings for 1 week before treatment. They were randomly divided into seven groups (13 animals per group). Four groups of rats were treated with different dosages (50, 100, 200, 400 mg/kg) of 2,5-HD *via* gavage, and one group was pretreated with Egb761 (100 mg/kg) *via* gavage 1 hour before 2,5-HD treatment (400 mg/kg). Two other control groups were respectively given with equivalent volume of normal saline (NS) and the same dose of Egb761. All animals were treated 5 days a week for 4 weeks. The use of animals and experimental protocols were approved by the Committee of Experimental Animal Welfare and Ethics of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention.

Neurological Testing

Toxicant-induced neurological deficits were quantified using the gait score[15]. The assigned scores were as follows: 1=normal gait; 2=slightly abnormal gait (tip-toe walking, hindlimb adduction); 3=moderately abnormal gait (obvious ataxia and foot spay with limb abduction); 4=severely abnormal gait (dragging hindlimbs, inability to support body weight and foot splay).

EB Assay and Fluorescence Microscopy

Protein extravasation, a marker of blood-nerve barrier (BNB) permeability, was evaluated by measuring the extravasation of EB. In brief, after anesthesia by injection of pentobarbital 2% (50 mL/kg) into the abdominal cavity, EB (20 mg/mL in NS; 80 mg/kg) was injected into the femoral vein. Ninety minutes later, one rat per group was perfused through the heart with 4.0% paraformaldehyde in 0.03 mol/L sodium phosphate buffer, pH 7.4. The sciatic-tibial nerves were excised and pinned without stretching to expanded polystyrene. Fixation was continued by immersion in 4.0% paraformaldehyde

in 0.03 mol/L sodium phosphate buffer, pH 7.4, at 20-24 °C for 24 h. After fixation and embedding, 5 μm longitudinal sections of the sciatic nerve of each animal were observed with fluorescence microscopy (E400, Nikon) using an emission filter of 520-550 nm. The EB emitted a bright red fluorescence that was captured and imaged using a Nikon DS-5M-U1 CCD camera.

In addition to microscopy studies, we also quantified the EB content in the sciatic nerves. Six animals per group were perfused with NS, and the sciatic nerves were dissected and weighed. A portion was put in formamide (7 mL/g wet weight tissue at 60 °C for 24 h), while the rest was dried at 60 °C for 24 h. The level of EB extracted in formamide was determined by fluorimetric spectrophotometry with an excitation wavelength of 635 nm and an emission wavelength of 687 nm using a Synergy H4 Hybrid Microplate Reader (Biotek, USA). The results were plotted on a standard EB curve (0.125-8 μg/mL). The EB content of each sample was expressed as EB μg/g dry weight of tissue[16].

Isolation of Peripheral Nerve Myelin and Extraction of P_0

The sciatic nerves of two rats in each group were removed, pooled, frozen in liquid nitrogen, pulverized and homogenized in 0.25 mol/L sucrose and 1 μg/mL aprotinin at 4 °C. The homogenate was layered over 0.9 mol/L sucrose and centrifuged at 100 000 × g for 3 h at 4 °C. Myelin was collected from the interface between the two sucrose layers, homogenized in 10 mL ice-cold water, and centrifuged at 15 000 × g for 30 min at 4 °C. The myelin pellet was homogenized in 0.25 mol/L sucrose and 1 μg/mL aprotinin, separated again on a sucrose gradient, and rinsed twice in cold water as described above. The final pellet was diluted in a buffer containing 150 mmol/L sodium chloride, 1.2% NP-40, 1.2% Triton X-100, 0.1% SDS, 50 mmol/L Tris, pH 7.5, and protease inhibitors [2 mmol/L pefabloc, 1 μg/mL pepstatin, 1 μg/mL leupeptin (Amresco, Solon, OH, USA)][17], and incubated for 30 min at 4 °C. The mixture was centrifuged at 15 000× g for 10 min at 4 °C, and the total protein concentration of the supernatant was determined with a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). The supernatant was then resuspended in SDS sample buffer, boiled for 10 min and finally stored at -20°C.

Western Blotting

The total protein (1.5 μg per lane) from each sciatic nerve were separated by SDS-PAGE on a 12% acrylamide gel and transferred onto nitrocellulose membranes. After electroblotting and transfer, membranes were blocked for 1 h at room temperature. Incubation with primary goat polyclonal antibodies (1:2 000; Abcam, USA) overnight at 4 °C was followed by washing and treatment with horseradish peroxidase-conjugated anti-goat (1:10 000; Zhongshan Golden Bridge, China) secondary antibodies. The signal intensity was detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, California, USA), and the relative optical densities of the bands were quantified using the Umax Imaging Program and Image-Pro Plus software.

RNA Extraction and Real-time PCR

The P_0 mRNA content was analyzed by real-time PCR using the MyiQTM Single-Color Real-Time PCR Detection System (Bio-Rad). GAPDH was used as a house-keeping gene in this study. The sciatic nerves were ground into a powder with a pestle and mortar in liquid nitrogen, and the total RNA was isolated with RNAgents Isolation System (Promega A3100, Madison, WI, USA) according to the manufacturer's protocol. Total RNA was dissolved in 50 μL of nuclease-free water. The A260/A280 ratio was calculated to determine the RNA purity. After heating to 70 °C and then chilling on ice for 5 min, 8 μL of the RNA samples were subjected to 20 μL of the reverse transcription reaction buffer (Promega A3500). The mixture was incubated at 42 °C for 45 min, 95 °C for 5 min, and 5 °C for 5 min. PCR reactions were performed with 1 μL of the appropriate cDNA dilution in a final volume of 25 μL with iQTM SYBR Green Supermix (Bio-Rad). The reaction mixture contained 200 nmol/L of the P_0 primers or 100 nmol/L of the GAPDH primers. The P_0 primers sequences used were 5'-TGT TGC TGC TGT TGC TCT TC-3' and 5'-TTG GTG CTT CGG CTG TGG TC-3' (181 bp). The P_0 PCR parameters were 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 90 s, and finally 72 °C for 10 min[18]. The GAPDH primers used were 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3' (452 bp). The GAPDH PCR parameters were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 1 min, and 68 °C for 2 min, and finally 68 °C for 10 min. Relative quantification was carried out using the comparative Ct method. The amount of target mRNA was normalized to an endogenous reference and, relative to a calibrator, is given by $2^{-\triangle\triangle Ct}$.

Statistical Analysis

All results are presented as the mean and Standard Error (SE). Body weight changes were analyzed using one-way ANOVA, followed by the Student-Newman-Keuls (SNK) test. All other results were analyzed using the Kruskal-Wallis H rank test. $P<0.05$ was considered to be of statistical significance.

RESULTS

Body Weight Changes and Neurological Evaluation

Rats injected with NS and Egb761 showed steady body weight gain. However, exposure to 2,5-HD led to a significant decrease in body weight gain. Rats administered with 200 mg/kg exhibited slight gait abnormalities from the 4th week and had a mean gait score of 1.23±0.12. Rats exposed to 400 mg/kg 2,5-HD showed more progressive gait abnormalities and their body weight began to decrease from the 2nd week. At the end of the 4th week, these rats were completely unable to lift their hindquarters or to rear, and had a mean gait score (3.77±0.12) that was significantly higher than the NS group ($P<0.01$). Egb761 pretreatment significantly reduced the loss of body weight ($P<0.01$) and mitigated the gait abnormalities (2.85±0.22) induced by 400 mg/kg 2,5-HD ($P<0.01$). The body weight and gait score changes are shown in Table 1 and Figure 1.

Table 1. Effect of 2,5-HD Dosage on Rat Weight Gain (g) ($\bar{x}$±SE)

Group (mg/kg)	0 w	1 w	2 w	3 w	4 w
NS	218.9±2.6	302.0±4.0	338.2±3.7	370.4±4.5	397.0±4.6
50	219.1±2.4	289.8±3.7*	328.7±6.9	350.2±5.7*	372.2±6.5**
100	218.9±3.1	289.5±4.2*	311.7±4.3**	339.0±4.4**	353.7±4.3**
200	218.8±2.9	286.5±4.0*	305.0±4.5**	329.9±5.1**	335.6±6.3**
400	218.0±2.3	259.0±2.4**	225.9±5.0**	221.8±9.5**	219.7±6.5**
Egb761	218.8±2.5	295.6±4.6	326.6±6.1	359.7±7.0	384.2±7.8
Egb761+2,5-HD	218.0±2.8	264.7±4.0	271.1±5.3$^{\#}$	268.3±7.8$^{\#}$	274.9±6.0$^{\#}$

Note. *: $P<0.05$, **: $P<0.01$, compared with the NS group; $^{\#}$: $P<0.01$, compared with the 400 mg/kg group.

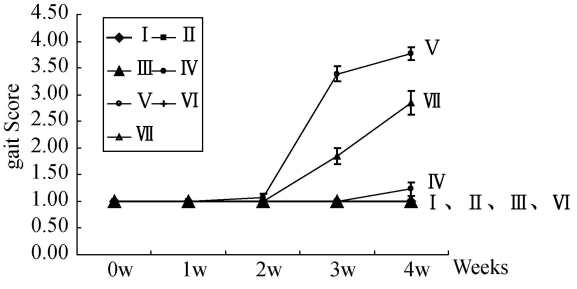

Figure 1. Changes in the gait score and corresponding time-matched control rats over 4 weeks of 2,5-HD treatment. Values are mean±SE (n=13). I: NS; II: 50 mg/kg; III: 100 mg/kg; IV: 200 mg/kg; V: 400 mg/kg; VI: Egb761 (100 mg/kg); VII: Egb761 (100 mg/kg) + 2,5-HD (400 mg/kg).

Distribution of Fluorescence in Longitudinal Sections of Sciatic-tibial Nerves

Relatively weak EB fluorescence was observed in longitudinal sections of the sciatic-tibial nerve in the NS and Egb761 control groups (Figures 2a and 2f). In 2,5-HD intoxicated rats fluorescence was readily observed, however, with fluorescence most intense for the group administered with 400 mg/kg (Figure 2e). Egb761 attenuated the increase of vascular permeability induced by 2,5-HD (Figure 2g).

EB Levels in Sciatic-tibial Nerves

Six rats in each group were tested. A clear but non-significant tendency was observed (Figure 3). The EB level in sciatic-tibial nerves of the NS group was similar to that observed for the Egb761 control group. Treatment with 2,5-HD increased EB extravasation. However, pretreatment with Egb761 reduced the EB level to a small extent, and statistical analysis gave a negative result ($P>0.05$).

P_0 Protein Expression in Sciatic Nerve Myelin

To investigate changes in the expression of P_0 in the sciatic nerve myelin of rats treated with 2,5-HD, goat polyclonal anti-P_0 antibody (Abcam, Cambridge, UK) was used to examine protein levels by Western blotting. As shown in Figure 4, the level of P_0

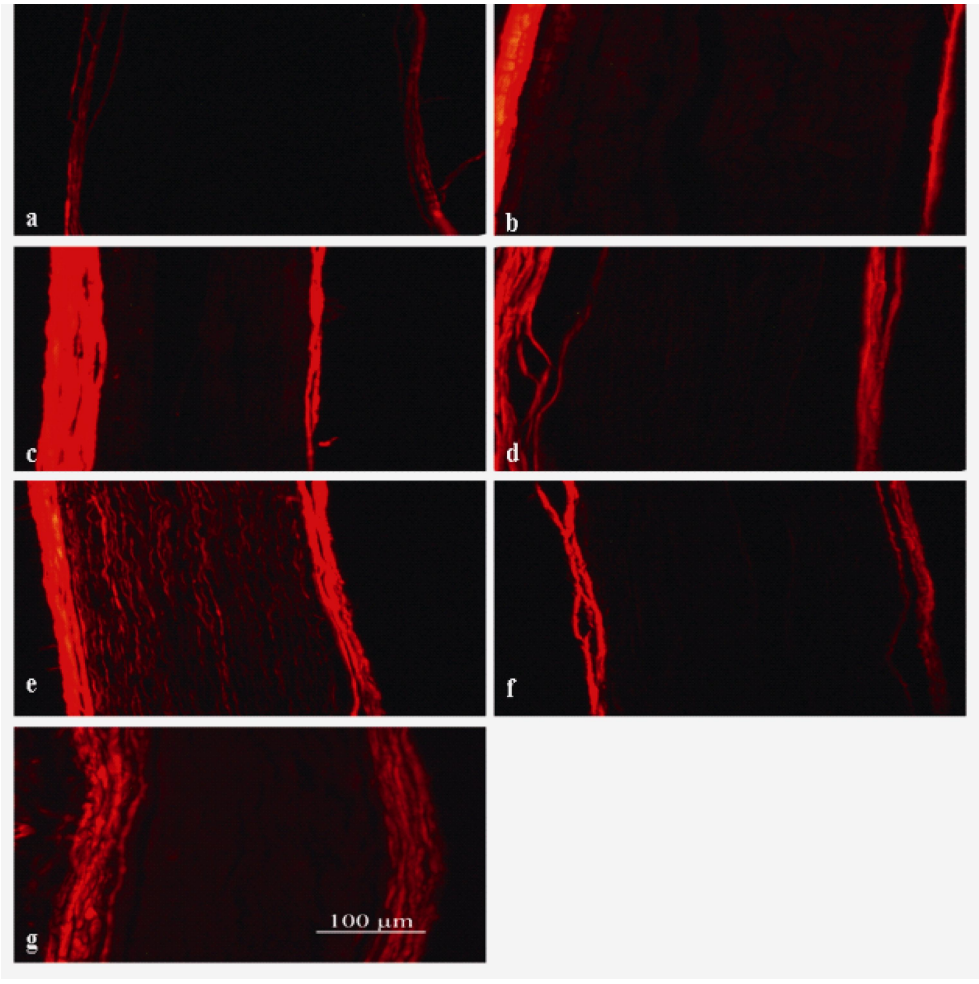

Figure 2. Effect of 2,5-HD on the blood-nerve barrier permeability of the sciatic nerve as measured by fluorescence microscopy. a: NS; b: 50 mg/kg; c: 100 mg/kg; d: 200 mg/kg; e: 400 mg/kg; f: Egb761 (100 mg/kg); g: Egb761 (100 mg/kg) + 2,5-HD (400 mg/kg). Sections of the sciatic nerves were observed with fluorescence microscopy using an emission filter of 520-550 nm, and the bright red fluorescence emitted by EB was captured (200×).

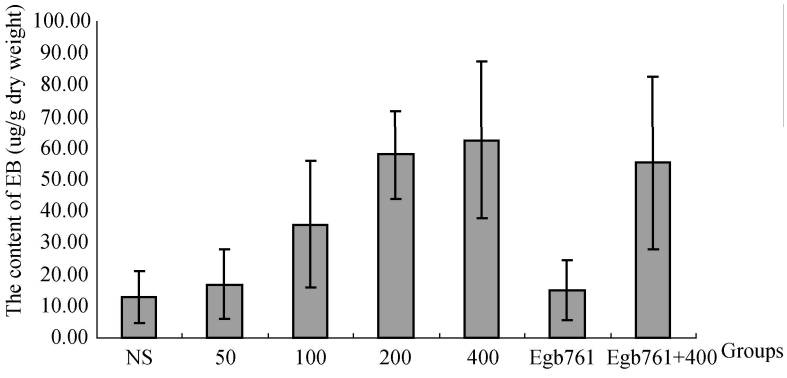

Figure 3. Changes in the EB content in sciatic nerves with 2,5-HD dosage. EB extracted in formamide from each sample was quantified by fluorimetric spectrophotometry and was expressed as EB μg/g dry weight of tissue. Values are mean±SE (*n*=6).

decreased in rats treated with 2,5-HD compared with the control NS group, with the most notable changes observed for the group administered with 400 mg/kg. P_0 expression decreased in a 2,5-HD dose-dependent manner. In comparison with the 400 mg/kg group (V), Egb761 pretreatment (VII) significantly inhibited the reduction in P_0 expression induced by 2,5-HD (P<0.05).

Changes in P_0 mRNA Expression

To determine the effect of 2,5-HD exposure on P_0 gene expression, the mRNA of P_0 was examined in four rats from each group. The expression of P_0 mRNA was normalized to that of the GAPDH gene. In this study, the PCR efficiency of P_0 and GAPDH were 103.1% and 96.0%. As shown in Figure 5, 2,5-HD exposure led to a decrease in the expression of the P_0 gene in a dose-dependent manner. In the 400 mg/kg group, 2,5-HD exposure reduced the expression of the P_0 gene by 77% when compared to expression levels in the NS group. Egb761 pretreatment did not significantly increase P_0 gene expression relative to the 400 mg/kg group (P>0.05).

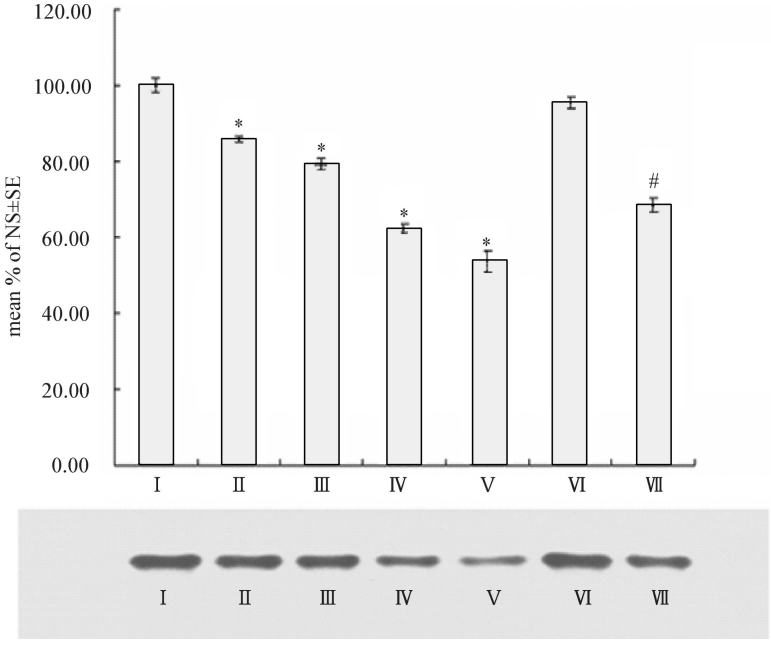

Figure 4. P_0 protein expression in sciatic nerves of 2,5-HD treated and control rats (n=4). I: NS; II: 50 mg/kg; III: 100 mg/kg; IV: 200 mg/kg; V: 400 mg/kg; VI: Egb761 (100 mg/kg); VII: Egb761 (100 mg/kg) + 2,5-HD (400 mg/kg). P_0 content was determined using western-blotting technology and expressed as the mean percentage of NS (%)±SE. Representative immunoblots are shown below the graph. Significant statistical difference is indicated by $^{*}P$<0.05 for the NS group, and $^{\#}P$<0.05 for the 400 mg/kg group.

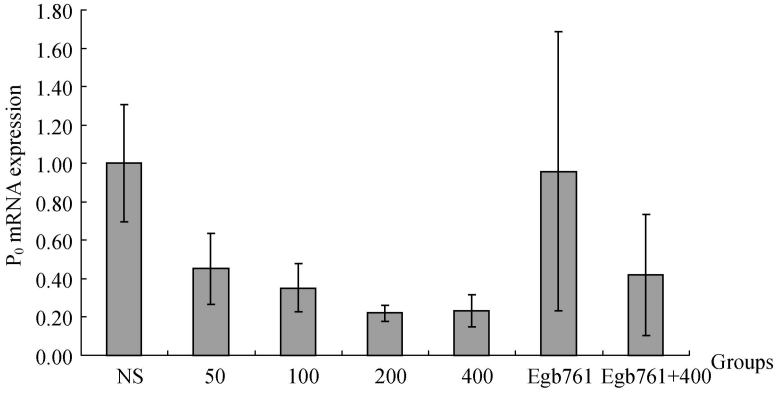

Figure 5. Real-time PCR analysis P_0 mRNA levels following exposure to 2,5-HD. GAPDH was used as an endogenous reference. Relative quantification was carried out using the comparative Ct method. Values are mean±SE (n=4).

DISCUSSION

Chronic exposure to *n*-hexane can result in both sensory and motor dysfunction, and induces a symmetrical and distal sensorimotor neuropathy[19-20]. Neuropathological lesions that occur include retraction of myelin from the nodes of Ranvier and segmental demyelination, as well as distal axonal Wallerian-type degeneration[21]. Consistent with previous reports, our results show that body weight gain is significantly hampered by 2,5-HD treatment, and neurotoxicity symptoms worsen progressively during treatment, based on the mean scores of neurological evaluation measured. The peripheral nerve toxicity triggered by 2,5-HD also had an apparent dose-dependency.

BNB can restrict the entry of blood-borne and water-soluble substances into endoneurial compartments and maintains homeostasis in the endoneurial microenvironment of the peripheral nerve. Our previous study showed that the permeability of the BNB increased with 2,5-HD treatment[22]. This raised the possibility that 2,5-HD passes through endoneurial blood vessels and produces toxic effects directly in the peripheral nerve, including in the myelin.

P_0 constitutes more than 50% of the total peripheral myelin protein in vertebrates and is the major adhesive and structural element of peripheral myelin. P_0 was selected for investigation of the mechanism of peripheral nerve toxicity induced by 2,5-HD. Our results demonstrate that 2,5-HD intoxication is associated with a reduction of both P_0 mRNA and protein in the peripheral nerve myelin. The P_0 mRNA decreased in a dose-dependent manner, with a decrease of 77% compared to the control group observed when the maximum dose tested (400 mg/kg) was administered. The decrease in P_0 gene expression observed may also explain the decreased levels of P_0 protein in the myelin, as measured by western blot. In this study, the P_0 protein contained in the blots was quantified and normalized relative to the total amount of protein loaded for each sample. We were unable to use a routine internal loading control due to the loss of housekeeping proteins such as GAPDH during the purification process. Previous reports have shown that the expression of many other proteins, including *β*-actin, and α- and *β*-tubulin, are affected by 2,5-HD[23]. Our study is consistent with previous studies that indicate that changes in P_0 levels can lead to various myelin related diseases, such as congenital hypomyelination[24] and autoimmune peripheral neuropathy[25]. Since P_0 is a major adhesive and structural element of peripheral myelin, the significant reduction observed after 2,5-HD treatment may lead to the functional disturbance of myelin and explain the characteristics of the neuropathology observed in 2,5-HD-induced peripheral neuropathy.

Egb761 can prevent neuronal death caused by a variety of mechanisms, and has protective effects against various central nervous system disorders including neurodegenerative diseases. To date, only a few studies have focused on the effect of Egb761 on peripheral nerve function. The aim of the present study was to investigate the potential protective effect of Egb761 on the toxic peripheral neuropathy caused by 2,5-HD. In this study, Egb761 pretreatment attenuated increases in BNB permeability caused by treatment with 400 mg/kg 2,5-HD, as measured by fluorescence intensity and the level of EB in sciatic-tibial nerves. The expression of P_0 mRNA was higher in a group pretreated with Egb761. Furthermore, our study found that Egb761 pretreatment could significantly reduce the loss of body weight (P<0.01) and mitigate the gait abnormalities (P<0.01) observed in the high-dose 2,5-HD group that did not have pretreatment (Figure 1). This indicates that Egb761 may have potential in alleviating the symptoms of 2,5-HD-induced peripheral neuropathy in rats. This is the first direct demonstration of the protective effect of Egb761 on 2,5-HD-induced toxic peripheral neuropathy.

2,5-HD is a diketone electrophile that reacts covalently with nucleophilic lysine ε-amine groups to form 2,5-dimethylpyrrole adducts on neurofilaments (NFs) and other proteins[26]. Once formed, the pyrrole adducts can undergo additional oxidative reactions that result in crosslinked proteins. These reactions are consistent with the well-known susceptibility of alkylpyrroles to auto-oxidation. This phenomenon is minimized when an atmosphere of argon or nitrogen is applied, and in the presence of free radical scavengers[27]. Recently, it has been verified that 2,5-HD can induce reactive oxygen species generation and mitochondrial damage[28]. Egb761 has been shown to increase protein levels and activities of antioxidant enzymes such as superoxide dismutase and catalase in the rat hippocampus[29] and glutathione (GSH) reductase in mouse liver[30]. It can also enhance the activity of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme of GSH synthesis[31]. Furthermore, Egb761 is a particularly potent inhibitor of oxidative stress in the mitochondria.

Mitochondrial DNA is a major target of free-radical attack[32].

2,5-HD can also cause increases in intracellular Ca^{2+} concentrations[28]. Studies have established the important role of Ca^{2+} accumulation in injured axons[33-34]. Enhancement of intracellular Ca^{2+} can induce increased levels of calmodulin (CaM), Ca^{2+}/CaM-dependent protein kinase II (CaMKII) and protein kinase C (PKC), which are positively correlated with gait abnormality[35]. Many studies have indicated that Egb761 can modify ion imbalance and reduce Ca^{2+} accumulation[36-38].

In conclusion, this study indicates that 2,5-HD can inhibit the expression of P_0 in a dose-dependent manner, and that these changes in P_0 expression may play an important role in the toxic peripheral neuropathy induced by 2,5-HD. Egb761 has been shown to have a protective effect on 2,5-HD-induced peripheral neurotoxicity in rats, and may therefore have potential as a therapeutic strategy to counteract the detrimental effects of 2,5-HD. The mechanism of this protective action remains to be elucidated.

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