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Research Article

Ginseng gintonin alleviates neurological symptoms in the G93A-SOD1 transgenic mouse model of amyotrophic lateral sclerosis through lysophosphatidic acid 1 receptor

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ABSTRACT

Background: We recently showed that gintonin, an active ginseng ingredient, exhibits antibrain neurodegenerative disease effects including multiple target mechanisms such as antioxidative stress and antiinflammation via the lysophosphatidic acid (LPA) receptors. Amyotrophic lateral sclerosis (ALS) is a spinal disease characterized by neurodegenerative changes in motor neurons with subsequent skeletal muscle paralysis and death. However, pathophysiological mechanisms of ALS are still elusive, and therapeutic drugs have not yet been developed. We investigate the putative alleviating effects of gintonin in ALS.

Methods: The G93A-SOD1 transgenic mouse ALS model was used. Gintonin (50 or 100 mg/kg/day, p.o.) administration started from week seven. We performed histological analyses, immunoblot assays, and behavioral tests.

Results: Gintonin extended mouse survival and relieved motor dysfunctions. Histological analyses of spinal cords revealed that gintonin increased the survival of motor neurons, expression of brain-derived neurotrophic factors, choline acetyltransferase, NeuN, and Nissl bodies compared with the vehicle control. Gintonin attenuated elevated spinal NAD(P) quinone oxidoreductase 1 expression and decreased oxidative stress-related ferritin, ionized calcium-binding adapter molecule 1-immunoreactive microglia, S100β-immunoreactive astrocyte, and Olig2-immunoreactive oligodendrocytes compared with the control vehicle. Interestingly, we found that the spinal LPA1 receptor level was decreased, whereas gintonin treatment restored decreased LPA1 receptor expression levels in the G93A-SOD1 transgenic mouse, thereby attenuating neurological symptoms and histological deficits.

Conclusion: Gintonin-mediated symptomatic improvements of ALS might be associated with the attenuations of neuronal loss and oxidative stress via the spinal LPA1 receptor regulations. The present results suggest that the spinal LPA1 receptor is engaged in ALS, and gintonin may be useful for relieving ALS symptoms.

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1. Introduction

Amyotrophic lateral sclerosis (ALS), which is also widely known as Lou Gehrig's disease, is a neurodegenerative disease which selectively targets spinal motor neurons [1]. Patients with ALS suffer deteriorating symptoms with progressive motor functional deficits by muscular degenerations and subsequent atrophy, eventually resulting in death [1]. Until now, although the main cause of ALS has not been elucidated, diverse pathological hallmarks, including aggregation of misfolded protein, mitochondrial dysfunctions, glutamatergic excitotoxicity, and neuronal inflammation, might be correlated with ALS occurrence [2]. The most abundant type of ALS is sporadic ALS with unknown causes, while about 20% of ALS cases are familial ALS with genetically inherited mutations [2,3].

Up to present time, studies regarding familial ALS have identified various causes of the disease, including mutations of superoxide dismutase 1 (SOD1) (also called Cu/Zn SOD) and hexanucleotide repeats in a noncoding region of C9orf72 [1,3]. The role of SOD1, an important intracellular antioxidant, is to convert superoxide radicals to hydrogen peroxide to relieve oxidative stress [4]. Overexpression of the human ALS-associated mutant form of SOD1 (G93A-SOD1) in transgenic mice exhibits multiple neurological symptoms that closely mimic those of human ALS, including spinal motor neuron losses [5]. Therefore, accumulating evidence suggests that oxidative stress is the main contributor in the pathophysiology of ALS [5]. Currently, several therapeutic strategies, including riluzole administration and stem cell-based therapies, have been attempted, but no prominent or feasible therapeutic advances have been discovered [6,7].

Panax ginseng Meyer is a traditional herbal medicine used as a tonic for the promotion of rejuvenation and longevity, as well as to treat frailty, stress, weakness, and fatigue, both mental and physical [8]. As a functional and medicinal food for brain health, ginseng is usually consumed for preventive purposes even in the absence of brain dysfunctions [8]. Traditionally, ginseng has been consumed by decoction with water. Recently, alcohol extraction has been used to obtain water-insoluble components from ginseng [9]. Previous studies have reported that crude ginseng total saponin fraction contains a novel component which was isolated and named gintonin, a nonacidic polysaccharide and a nonsaponin [10]. Lysophosphatidic acid (LPA), a biomarker component of gintonin, is a typical lysophospholipid showing pleiotropic effects, as a lipid-derived growth factor from embryonic brain development to adult brain and spinal functions in the central nervous system, including synaptic transmissions, neurogenesis, and cognitive functions [10]. Administered gintonin acts as an exogenous ligand of G protein–coupled LPA receptor. The gintonin primarily induces $[Ca^{2+}]_i$ transient through the LPA receptor signal transduction in neuronal cells [9]. Gintonin uses a second messenger Ca^{2+} as a tool for its diverse *in vitro* cellular effects from ion channel regulations to neurotransmitter release [9]. In addition, *in vivo* preclinical studies have found that oral administration of gintonin alleviates symptoms of neurodegenerative brain diseases, such as Alzheimer disease (AD) by improving cognitive functions and attenuating cortex and hippocampal amyloid plaque accumulations, Parkinson disease (PD) by improving abnormal motor functions by increasing nigrostriatal dopaminergic neurons under insults of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and Huntington disease (HD) by ameliorating neurological impairment and 3-nitropropionic acid-induced striatal toxicity [11–16]. Interestingly, the underlying molecular mechanisms for gintonin-mediated antineurodegenerative diseases comprise LPA receptor regulations, antioxidation, and antiinflammatory effects.

However, relatively little is known regarding the effects of ginseng against ALS. Therefore, by using a G93A-SOD1 transgenic mouse model of ALS, we aimed to investigate whether long-term oral administration of gintonin can alleviate the neurological symptoms of ALS in functional and structural aspects. Here, we showed that the administration of gintonin alleviated motor dysfunctions by increasing the survival of spinal motor neurons and spinal cord expressions of brain-derived neurotrophic factors (BDNFs) and choline acetyltransferase (ChAT) compared with the control vehicle and decreasing spinal cord oxidative stress-related Fe^{2+}/ferritin, ionized calcium-binding adapter molecule 1 (Iba1)-immunoreactive microglia, and S100β-immunoreactive astrocytes, as well as Olig2-immunoreactive oligodendrocytes. In addition, gintonin significantly attenuated the elevated expression of NAD(P)H quinone oxidoreductase 1 (NQO1) and restored the decreased LPA1 receptor level in the spinal cord. For the first time, these results show that the spinal LPA1 receptor is involved and that oral gintonin administration restored *in vivo* neurological symptoms in the ALS animal model. We also further discuss the molecular mechanisms regarding how long-term oral administration of gintonin alleviates neurological symptoms of ALS.

2. Materials and methods

2.1. Animals

Transgenic G93A-SOD1 male mice which present human ALS phenotype via G93A mutation in SOD1 [17] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed singly in a standard clear plastic cage under the controlled environment of temperature (23~24°C) and humidity (50~60%). The mice were maintained under a 12 h light and 12h dark cycle and fed food (Purina 5008, Purina Korea, Korea) and tap water ad libitum. The use and care of the mice follow the guidelines established to comply with current international laws and policies (National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Konkuk University (Permit Number: 16-206). All experimental procedures were conducted in a manner that reduced the number of animals used and minimized the distress from the present study.

2.2. Gintonin preparation

The gintonin-enriched fraction was prepared as described previously [18]. In total, 1 kg of four-year-old ginseng was used to gain extracts (350 g) which was further concentrated, dissolved in distilled, cold water, ethanol extracted, and centrifuged. After centrifugation, the precipitate was lyophilized and called as the gintonin-enriched fraction [18]. Briefly, ginseng gintonin is an isolated glycolipoprotein fraction which consists of carbohydrates, lipids, proteins, and other minor components. The main active components of gintonin are lysophospholipids such as LPAs. Liquid chromatography-mass spectrometry/mass spectrometry analysis of lipid compositions of gintonin showed that fatty acids (7.53% linoleic acid, 2.82% palmitic acid, and 1.46% oleic acid), 0.60% lysophospholipids and phospholipids, and 1.75% phosphatidic acids are detected [18].

2.3. Gintonin treatment

Gintonin dosages for long-term treatments to mice were adopted from design as described in our previous studies [14–16,19]. G93A-SOD1 mice were divided into four experimental groups: saline only (wild-type; n = 8, and Tg; n = 10) or gintonin (50 or 100 mg/kg/day) (Tg + GT50 and Tg + GT100; n = 10, each). Gintonin was suspended with saline before administration and prepared fresh every day. G93A-SOD1 mice were orally administered saline or gintonin from seven to 18 weeks of age (Fig. 1A).

2.4. Body weight measurement

During experiments, body weight of each mouse was checked on Monday morning of every week until the end of the experiment.

2.5. Neurological score assessment

To assess the neurological score of each mouse, the health condition of the animals was assessed according to the previous study [20]. In particular, depending on the degree of movement and paralysis of each hindlimb, neurological stages (from 0 to 4) were assigned (summary provided in Table 1). At Stage 3, G93A-SOD1 mice were partially paralyzed with forward movement, while mice at Stage 4 had completely paralyzed forelimbs and hindlimbs without forward movement.

2.6. Evaluation of survival rate

At Stage 4, the hindlimb and forelimb of G93A-SOD1 mice became completely paralyzed and death followed within a day. The time of death was defined as the date on which these neurological symptoms were observed.

2.7. Rota-rod test

The rota-rod test was conducted to assess motor coordination and balance as described in the previous study [21]. Briefly, mice were trained to stay on the rotating rod (Dae-Jong Co. Inc., Seoul, Korea) and tested trained mice at a constant rotation speed of 16 rpm in 300s. The time to fall off from the rotating rod motor was measured as latency. Mice were given three trials with 15 min intertrial intervals, and the longest latency to fall was recorded.

2.8. Tissue processing and histological analyses

For histology, wild-type, G93A-SOD1 alone, G93A-SOD1 + gintonin (50 mg/kg, GT50), and G93A-SOD1 + gintonin (100 mg/kg, GT100) groups (n = 5 in wild-type, Tg, GT50, GT100 groups) were anesthetized with 1.5 g/kg urethane (Sigma-Aldrich, St. Louis, MO, USA). Using 0.1 M phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), each mice was transcardially perfused and fixed. Then, lumbar spinal cords were carefully dissected out and postfixed in the same fixative for two days. As described in previous studies [19,22], histological procedures were performed. Briefly, paraffin blocks of lumbar spinal cord tissues (L3~5) were prepared. Then, serially cut 5-µm-thick sections (microtome, Leica, Wetzlar, Germany) were

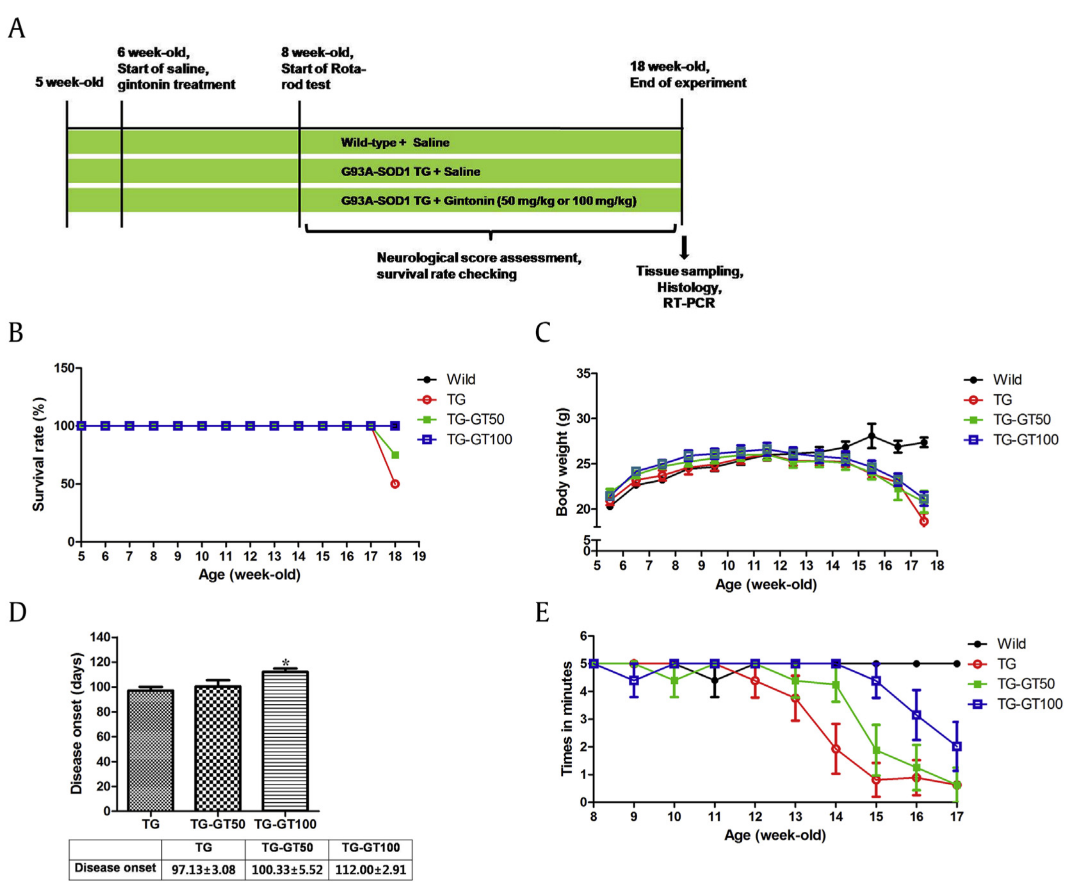

Fig. 1. Effects of gintonin on survival rate, body weight, rota-rod test, and onsets of neurological symptoms in G93A-SOD1 TG mice. (A) Experimental design. (B) Survival rate between the saline- and gintonin-treated groups. (C) Change of body weight during the experimental period of five-week-old mice, (D) summary histograms on the time delay of onset of neurological signs to hindlimb paralysis (neurological stage 3), and (E) motor functional analysis by the rota-rod test in saline-alone G93A-SOD1 (TG), G93A-SOD1 + gintonin (50 mg/kg, GT50), and G93A-SOD1 + gintonin (100 mg/kg, GT100) groups. The mean body weights of each groups were weekly measured and checked from 5 weeks to 18 weeks (n = 8-10 per group). *P < 0.05, indicating a significant difference compared with saline-treated G93A-SOD1 TG mice. Data are presented as means ± standard error of the mean.

Table 1
Neurological Stages (NS) were assigned by clinical signs

NS1	The hindlimb presents an abnormal splay, that is, it is collapsed or partially collapsed toward lateral midline or it trembles during tail suspension or it is retracted/clasped.	Normal or slightly slower gait.
NS2	The hindlimb is partially or completely collapsed, not extending much. There might still be joint movement.	The hindlimb is used for forward motion and any part of the foot is dragging along cage bottom.
NS3	Rigid paralysis in the hindlimb or minimal joint movement.	Forward motion however the hindlimb is not being used for forward motion.
NS4	Rigid paralysis in the hindlimbs.	No forward motion.

mounted onto coating slides (Muto-glass, Tokyo, Japan). Three slides per mouse were selected for each stain (total of 15 paraffin sections per group). Nissl staining and immunohistochemistry for marker proteins was conducted as previously described in our study [23,24]. Antigen retrieval with citrate buffer (pH 6.0) was conducted in a microwave oven (three heating cycles of 5 min each). PBS washed sections were then sequentially quenched with 0.3% hydrogen peroxide (H_2O_2) and blocked with 10% normal horse serum. Thereafter, the sections were incubated with primary antibodies (overnight at 4°C) against NeuN (1:1000; Millipore, Billerica, MA, USA), BDNF (1:500; Abcam, Cambridge, UK), ChAT (1:2000; Abcam), Iba1 (1:1000; Wako, Osaka, Japan), nuclear factor erythroid 2-related factor 2 (Nrf2, 1:250; Abcam), S100β (1:1000; Millipore), Olig2 (1:500; R&D systems, Minneapolis, MN, USA), or ferritin (1:250; Abcam). Subsequently, sections were exposed to biotinylated IgG (1:200; Vector, Burlingame, CA, USA) and streptavidin peroxidase complex (1:200; Vector). They were then visualized by the reaction with 3,3′-diaminobenzidine tetrachloride (Sigma-Aldrich) in 0.1 M Tris-HCl buffer (pH 7.2). Finally, sections were dehydrated and coverslipped in a mounting medium (Richard-Allan Scientific, Thermo Scientific).

2.9. Iron staining

As described in our previous study [24], deparaffinized sections were washed in deionized water (30 min) and incubated in Perl's solution (1:1; 2% HCl, 2% potassium ferrocyanide, Sigma–Aldrich) at room temperature (30 min). Then, sections were washed again and visualized using 0.5% 3,3′-diaminobenzidine tetrachloride (Sigma-Aldrich) in PBS (pH 7.4) for at least 15 minutes. Finally, sections were thoroughly washed and mounted.

2.10. Quantification of histological analyses

All histological quantification processes were conducted by an investigator blinded to the experimental group. The method used in this study was slightly modified from our previous study [19,23]. The slides were observed using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) connected to a PC monitor. To evaluate the effects of gintonin on G93A-SOD1 mice, the regions of interest in the spinal cord were analyzed. For histological analysis, the number of Nissl stained neurons, NeuN-, BDNF-, ChAT-, Iba1-, S100β-, Olig2-, or ferritin-immunoreactive cells in the ventral horn of the lumber spinal cord was counted using an image analysis system equipped with a computer-based charge-coupled device camera (Optimas 6.5 software, CyberMetrics, Scottsdale, AZ, USA).

In addition, the analysis of the Nrf2 and iron immunoreactivity in the gray matter of the ventral horn of the lumbar spinal cord was performed using ImageJ 1.50 software (NIH, Bethesda, MD, USA). The intensity of Nrf2 and iron was evaluated by relative optical density (ROD), which was obtained by transforming the mean gray level using the formula: ROD = log (256/mean gray level). Images were calibrated into an array of 512 × 512 pixels, and each pixel resolution was 256 gray levels. ROD of the background was determined in unlabeled portions of the sections for correction. A ratio of the ROD was expressed as the percentage of wild type.

2.11. Western blotting analysis

As described in previous study [14], the dissected lumbar spinal cords from each group (n = 3-5/group) were homogenized in lysis buffer, and samples were heated at 95°C for 5 min. Then, proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated with rabbit anti-LPAR1 (1:1,000; Abcam), rabbit anti-LPAR3 (1:1,000; Abcam), Nrf2 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or heme oxygenase-1 (HO-1, 1:1000; Enzo Life Sciences, Farmingdale, NY, USA) followed by horseradish peroxidase–conjugated secondary antibody. Band signals were visualized using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Glyceraldehyde-3-phosphate dehydrogenase was used as a control. Immunoblot images were analyzed and quantified using ImageJ 1.50 software (NIH).

2.12. Statistical analysis

As described in our previous study [19], data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and presented as means ± standard error of the mean. Two-sample comparisons were carried out using Student *t* test, while multiple comparisons were made using one-way analysis of variance followed by the Newman–Keuls multiple range test. The statistical significance threshold was set at $p < 0.05$.

3. Results

3.1. Oral administration of gintonin improves motor activity, delays ALS onset, and increases the survival rate in G93A-SOD1 Tg mice

This experiment was performed according to the experimental design described in Fig. 1A. Some saline-only treated G93A-SOD1 Tg mice began to die at around 17 weeks. At 18 weeks, the survival rate of the saline-only treated G93A-SOD1 Tg mice was 50%, while the survival rates of gintonin-treated G93A-SOD1 Tg mice with 50 and 100 mg/kg/day were 75% and 100%, respectively. Therefore, oral gintonin administration extended the survival of G93A-SOD1 mice (Fig. 1B). During the period of oral gintonin administration (aged from 7 to 18 weeks), body weights weekly measured in saline-only treated G93A-SOD1 mice were slightly lower than those of gintonin-treated G93A-SOD1 Tg mice, but there was no significant difference among saline-only treated and gintonin-treated G93A-SOD1 mice. At the end of the experiment, the difference in mean body weights (18-week-old mice) among four different groups was apparent: wild-type 27.35 ± 1.5 g, TG 18.61 ± 2.6 g, GT50 20.78 ± 2.3 g, and GT100 21.12 ± 2.3 g (Fig. 1C). When various neurological symptoms were estimated, as described in Table 1, G93A-SOD1 Tg mice usually showed an onset after 97.1 ± 3.1 days, as shown in Fig. 1D. However, gintonin-treated G93A-SOD1 Tg mice began to show neurological symptoms after

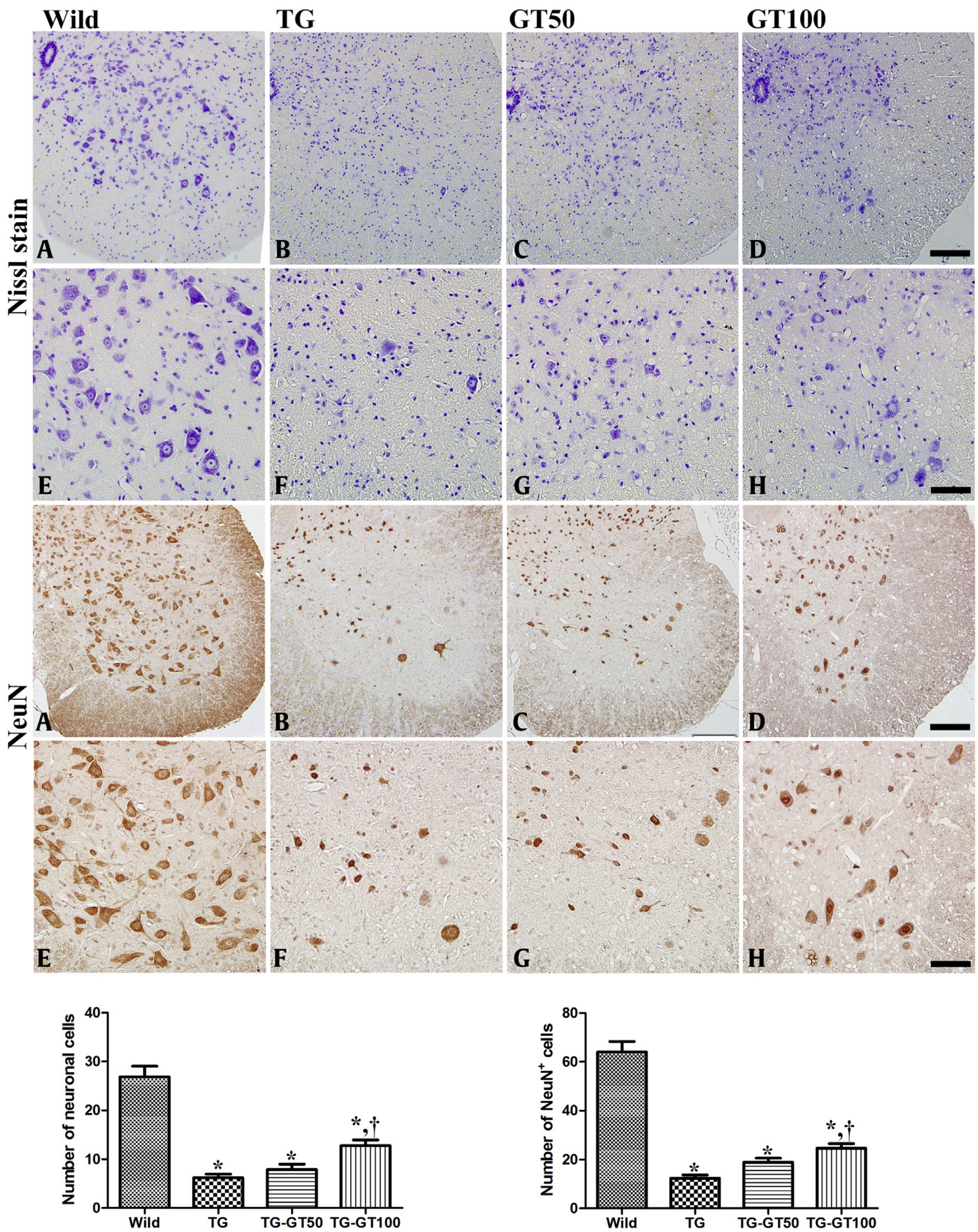

Fig. 2. Effects of gintonin on spinal neurons in G93A-SOD1 TG mice. (A to H) Nissl staining and immunohistochemistry for NeuN in the lumbar spinal cord of wild-type (wild, A and E), G93A-SOD1 (TG, B and F), G93A-SOD1 + gintonin (50 mg/kg, TG + GT50, C and G), and G93A-SOD1 + gintonin (100 mg/kg, TG + GT100, D and H) groups. Scale bars = 100 μm (A, B, C, and D), 50 μm (E, F, G, and H). The intact neuronal cells and NeuN-immunoreactive neurons in the ventral horn of the lumbar spinal cord of wild, TG, TG + GT50, or TG + GT100 groups (n = 5 per group; *$P < 0.05$, indicating a significant difference compared with the wild group; †$P < 0.05$, indicating a significant difference compared with the TG group; #$P < 0.05$, indicating a significant difference compared with the GT50 group). Data are presented as means ± standard error of the mean.

100.3 ± 5.5 and 112.0 ± 3.0 days with 50 and 100 mg/kg/day, respectively (Fig. 1D). Therefore, these results show that the gintonin-mediated extension of the survival rate of G93A-SOD1 Tg mice may be due to amelioration of neurological symptoms by gintonin.

3.2. Oral administration of gintonin ameliorates motor function deficits in G93A-SOD1 Tg mice

The rota-rod test to estimate motor functions was performed every second day from eight weeks of age. We examined that motor performance on the rotating rod was deteriorated in saline-only treated G93A-SOD1 Tg mice (Fig. 1E). Moreover, motor performance of this group was progressively aggravated over time. The gintonin (50 or 100 mg/kg/day) administration to G93A-SOD1 Tg mice delayed the motor dysfunctions by 3 weeks compared with saline-only treated G93A-SOD1 Tg mice throughout the rota-rod test period. High doses of gintonin (100 mg/kg) were more effective than low doses (50 mg/kg) until 16 weeks. These results suggest that the gintonin administration attenuates ALS-related motor dysfunctions.

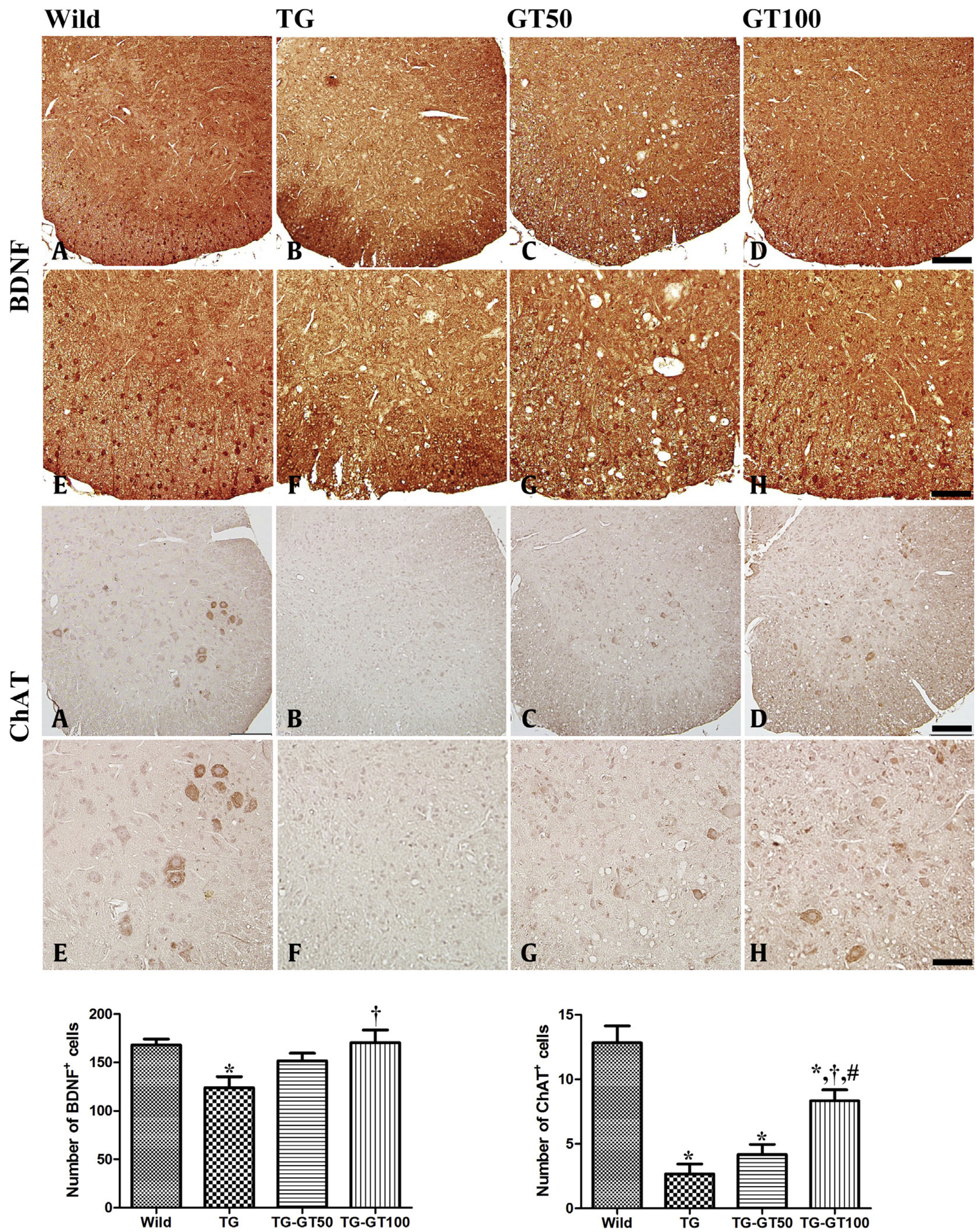

Fig. 3. Effects of gintonin on spinal BDNF and ChAT expressions in G93A-SOD1 TG mice. (A to H) Immunohistochemistry for BDNF and ChAT in the lumbar spinal cord of wild-type (wild, A and E), G93A-SOD1 (TG, B and F), G93A-SOD1 + gintonin (50 mg/kg, TG + GT50, C and G), and G93A-SOD1 + gintonin (100 mg/kg, TG + GT100, D and H) groups. Scale bars = 100 μm (A, B, C, and D), 50 μm (E, F, G, and H). The BDNF or ChAT-immunoreactive cells in the ventral horn of the lumbar spinal cord of wild, TG, TG + GT50, and TG + GT100 groups (n = 5 per group; *P < 0.05, indicating a significant difference compared with the wild group; †P < 0.05, indicating a significant difference compared with the TG group; #P < 0.05, indicating a significant difference compared with the GT50 group). Data are presented as means ± standard error of the mean. BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase.

3.3. *Oral administration of gintonin reduces neuronal loss in the spinal cords of G93A-SOD1 TG mice*

Next, we further conducted investigations to evaluate the effect of gintonin administration on neuronal loss in the spinal cords of G93A-SOD1 Tg mice. Nissl staining of neurons revealed that saline-only treated G93A-SOD1 Tg mice at 16 weeks had only 23.1% of the number of Nissl-stained neurons in the ventral horns of the spinal cords compared with that of wild-type mice. In contrast, gintonin-treated (50 or 100 mg/kg/day) G93A-SOD1 mice had an increased number of Nissl-stained cells by 126.8 ± 17.4% and 205.4 ± 18.9%, respectively, compared with saline-only treated G93A-SOD1 Tg mice (Fig. 2). NeuN immunohistochemistry also revealed that saline-only treated G93A-SOD1 Tg mice had 19.3 ± 2.0% of the number of NeuN-positive neurons in the ventral horns of the spinal cords compared with that of the wild-type mice, while gintonin-treated (50 or 100 mg/kg/day) G93A-SOD1 Tg mice exhibited an increase in the number of NeuN-stained neurons, 152.5 ± 13.8% and

199.0 ± 15.0%, respectively, to that in saline-only treated G93A-SOD1 Tg mice (Fig. 2). BDNF and ChAT immunohistochemistry revealed that saline-only treated G93A-SOD1 Tg mice had 73.7 ± 13.3% and 20.8 ± 5.9%, respectively, of the number of BDNF- and ChAT-positive cells in the ventral horns of the spinal cords compared with that of wild-type mice. Gintonin-treated (50 or 100 mg/kg/day) G93A-SOD1 mice exhibited an increase in the number of BDNF-positive cells, 122.5 ± 14.0% and 156.3 ± 35.2%, respectively, of that in saline-only treated G93A-SOD1 Tg mice. In addition, gintonin-treated (50 or 100 mg/kg/day) G93A-SOD1 Tg mice exhibited an increase in the number of ChAT-positive neurons, 137.7 ± 14.5% and 312.5 ± 59.9%, respectively, compared with saline-only treated G93A-SOD1 Tg mice (Fig. 3). Present results showed that the oral administration of gintonin increases neurotrophic factors, which supports the survival of neurons and synthesis of acetylcholine that are involved in the motor functions of G93A-SOD1 Tg mice.

3.4. Oral administration of gintonin reduces the glial marker proteins in the spinal cords of G93A-SOD1 TG mice

ALS induces changes in glial cells (astrocytes and microglia) and in neurons in the central nervous systems [5]. To evaluate the effect of oral gintonin administration on astrocyte and microglia, the marker proteins for microglia (Iba-1) and astrocytes (S100β) were immunostained and analyzed (Fig. 4; panel A). The number of Iba-1−immunoreactive microglia and S100β-immunoreactive astrocytes was significantly reduced in the spinal cord of the gintonin-treated G93A-SOD1 Tg mice compared with the saline-only treated G93A-SOD1 Tg group. These results indicate that oral gintonin administration attenuated the expression of marker proteins that were related to inflammatory responses in the lumbar spinal cord. The effects of gintonin on spinal oligodendrocyte changes in G93A-SOD1 Tg mice were also investigated as oligodendrocytes are important targets in the pathogenesis of ALS development [25]. A previous study reported that oligodendrocytes are initially increased in the mouse ALS model during disease progression [26]. Similarly, our present study showed an increase in oligodendrocytes in the lumbar spinal cord of G93A-SOD1 Tg mice. Interestingly, as shown in Fig. 4, the oral administration of gintonin decreased spinal cord injury-induced Olig2, a marker protein of oligodendrocyte, in the spinal cord, showing that gintonin might decrease oligodendrocytes in G93A-SOD1 Tg mice. Gintonin might attenuate the proliferation of oligodendrocytes in G93A-SOD1 Tg mice. These results indicate that gintonin administration provides beneficial effects on spinal glia through reduction of inflammatory marker protein expressions in addition to protection of spinal neurons in G93A-SOD1 Tg mice.

3.5. Oral administration of gintonin suppresses oxidative stress by decreasing ferritin expression and iron accumulations, Nrf2, heme oxygenase 1, and NQO1 expressions in the spinal cords of G93A-SOD1 Tg mice

If gintonin ameliorates the accumulation of oxidative stress in the spinal cords of G93A-SOD1 Tg mice was examined. Ferritin and iron accumulation were selected as markers for oxidative stress in the ALS animal model [27,28]. As shown in Fig. 5, saline-only treated G93A-SOD1 Tg mice showed increases of ferritin and iron staining. However, gintonin administration attenuated ferritin in the cell body and fibers and decreased intracellular iron accumulation. Then, the expression pattern of Nrf2, which is a transcriptional factor in the endogenous defense against oxidative stress, was examined. In the immunohistochemical study, spinal Nrf2

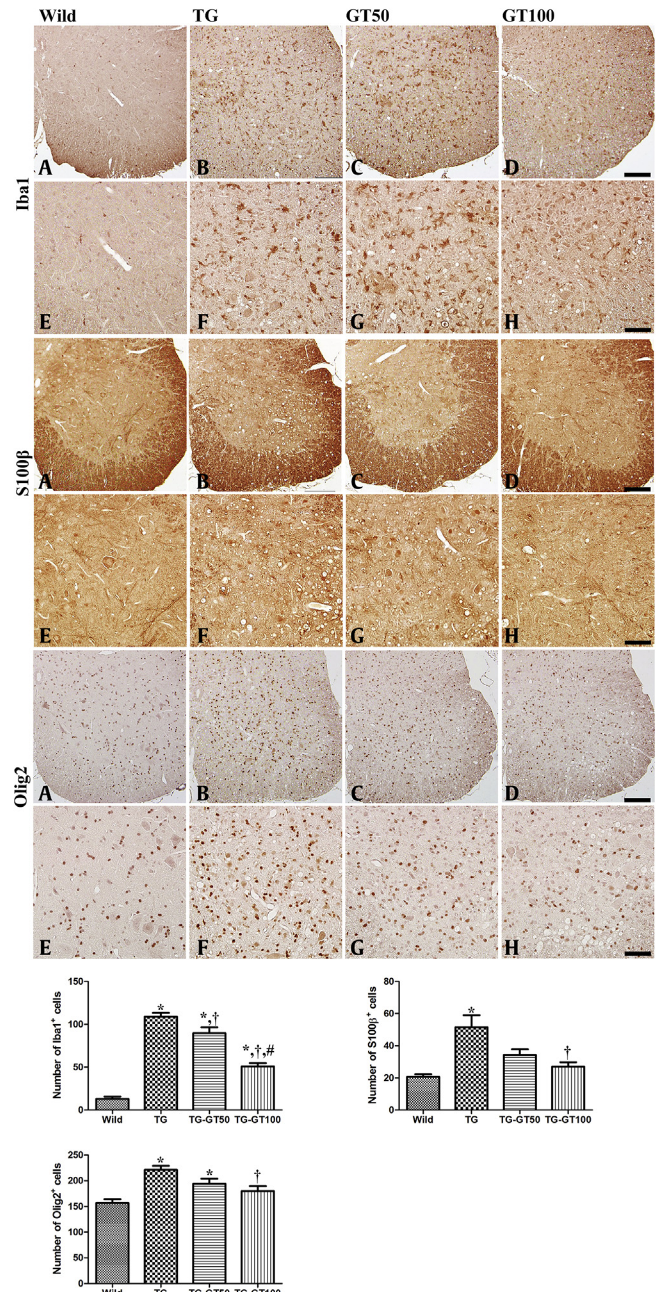

Fig. 4. Effects of gintonin on spinal Iba1, S100β, and Olig2 expressions in G93A-SOD1 TG mice. (A to H) Immunohistochemistry for Iba1, S100β, and Olig2 in the lumbar spinal cord of wild-type (wild, A and E), G93A-SOD1 (TG, B and F), G93A-SOD1 + gintonin (50 mg/kg, TG + GT50, C and G), and G93A-SOD1 + gintonin (100 mg/kg, TG + GT100, D and H) groups. Scale bars = 100 μm (A, B, C, and D), 50 μm (E, F, G, and H). The Iba1, S100β, or Olig2-immunoreactive glial cells in the ventral horn of the lumbar spinal cord of wild-type, TG, GT50, and GT100 groups (n = 5 per group; *P < 0.05, indicating a significant difference compared with the wild group; †P < 0.05, indicating a significant difference compared with the TG group; #P < 0.05, indicating a significant difference compared with the GT50 group). Data are presented as means ± standard error of the mean.

expression in saline-only treated G93A-SOD1 Tg mice showed an increase, whereas gintonin administration decreased spinal Nrf2 (Fig. 5). Similarly, in the immunoblot study, spinal Nrf2 protein levels in saline-only treated G93A-SOD1 Tg mice increased, whereas gintonin nonsignificantly attenuated the increase of Nrf2 expression (Fig. 6). The protein levels of spinal heme oxygenase 1

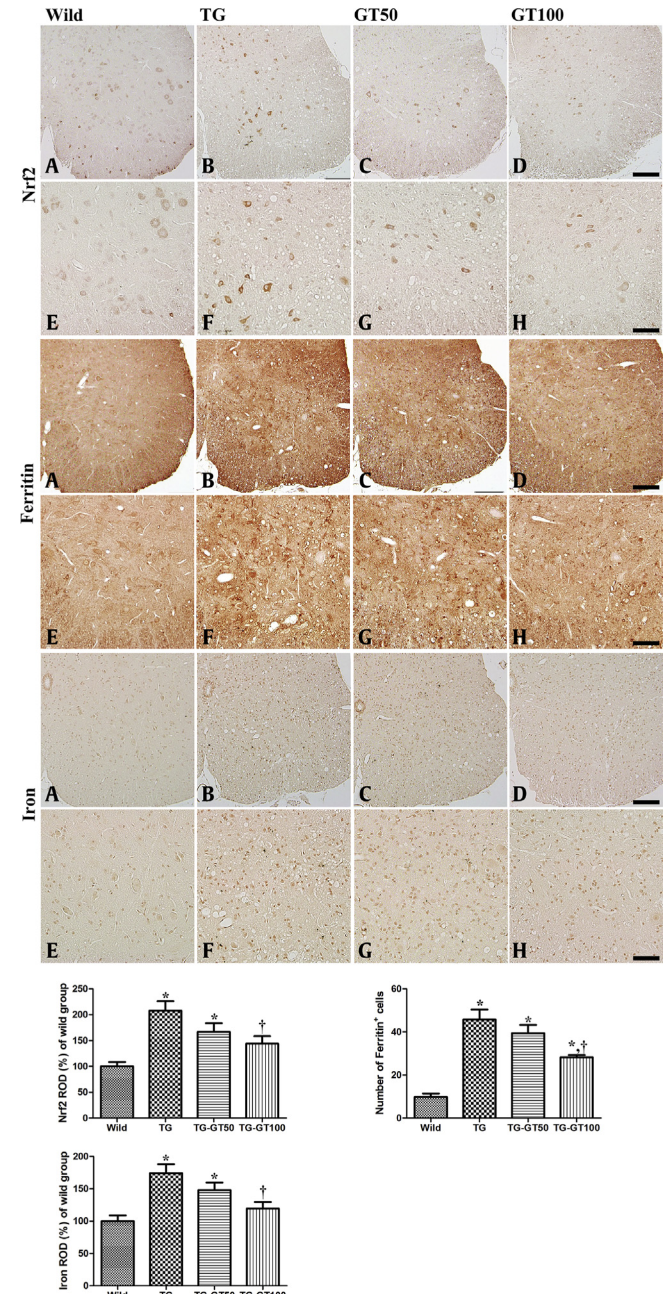

Fig. 5. Effects of gintonin on spinal Nrf2 expression and iron and ferritin staining in G93A-SOD1 TG mice. (A to H) Immunohistochemistry for Nrf2, iron staining, and immunohistochemistry for ferritin in the lumbar spinal cord of wild-type (wild, A and E), G93A-SOD1 (TG, B and F), G93A-SOD1 + gintonin (50 mg/kg, GT50, C and G), and G93A-SOD1 + gintonin (100 mg/kg, GT100, D and H) groups. Scale bars = 100 μm (A, B, C, and D), 50 μm (E, F, G, and H). The spinal Nrf2 expression and iron and ferritin staining in the ventral horn of the lumbar spinal cord of wild-type, TG, GT50, and GT100 groups (n = 5 per group; *$P < 0.05$, indicating a significant difference compared with the wild group; $^{\dagger}P < 0.05$, indicating a significant difference compared with the TG group; $^{\#}P < 0.05$, indicating a significant difference compared with the GT50 group). Data are presented as means ± standard error of the mean.

(HO1) also increased in saline-only treated G93A-SOD1 Tg mice, but gintonin treatment nonsignificantly reduced the protein levels of spinal HO1 (Fig. 6). In addition, the protein levels of spinal NQO1 also increased in saline-only treated G93A-SOD1 Tg mice; gintonin treatment significantly reduced the protein levels of spinal NQO1 (Fig. 6). These results show that the expressions of Nrf2/HO1/NQO1 increased in saline-only treated G93A-SOD1 Tg mice, and gintonin administration attenuated the increased expression level of Nrf2/HO1/NQO1.

3.6. *Oral administration of gintonin restored spinal LPA1 receptor expression levels that were decreased in G93A-SOD1 Tg mice*

In previous studies, it was observed that the gintonin-mediated amelioration of neurodegenerative diseases is mediated through LPA1/3 receptors [15,16]. Therefore, the changes in expression levels of LPA1/3 receptors in the spinal cords of G93A-SOD1 Tg mice after the oral administration of saline or gintonin were examined. As shown in Fig. 6, the protein expressional levels of the LPA1 but not the LPA3 receptor significantly decreased in saline-treated G93A-SOD1 Tg mice compared with wild-type mice. However, gintonin treatment restored spinal LPA1 receptor expression levels, similar to that in wild-type mice, indicating that ALS has effects on spinal LPA1 receptor levels, and gintonin administration prevents the reduction of spinal LPA1 receptors from ALS.

4. Discussion

In previous preclinical animal model studies, we have demonstrated that oral administration of gintonin ameliorated neurodegenerative brain diseases, such as Alzheimer disease, Parkinson disease, and Huntington disease [11–16]. Gintonin-mediated protection from brain-related neurodegenerative diseases is achieved via LPA1/3 receptor regulations, antioxidation, and antiinflammatory effects [11–16]. In this study, we further extended on whether gintonin can also ameliorate the neurological symptoms of ALS and prolong the life span in an ALS animal model. Here, it was shown that long-term oral administration of gintonin attenuated motor deficits and neuronal losses in the ventral horns of the spinal cords and increased the survival rate in G93A-SOD1 Tg mice (Figs. 1 and 2). This gintonin-mediated inhibition of spinal neuron loss was accompanied with a delay in the disease onset and mortality, as well as neurological symptoms by more than ten days (Fig. 1C–E), and finally extended the survival rate of G93A-SOD1 Tg mice (Fig. 1A). The present results indicate that gintonin protects against ALS-induced neurological symptoms and neurodegeneration in G93A-SOD1 Tg mice.

Therefore, long-term oral administration of gintonin exhibits beneficial effects on spinal neurons, as well as spinal glial cells, such as astrocytes, microglia, and oligodendrocytes, which could be a functional basis for the attenuation of neurological symptoms and prolonged survival rates. It may be noteworthy to consider the molecular mechanisms in the gintonin-mediated attenuation of ALS. In previous reports, we reported that gintonin is an exogenous ligand for LPA receptors in the nervous system [11]. Based on the effects of gintonin on the nervous systems, there are at least four ways for gintonin to exert its ameliorating effects against ALS. Although the LPA1 receptor plays an important role in neurogenesis during early brain developmental period [29], recent reports have shown that the LPA1 receptor also plays crucial roles in adult brain such as synaptic transmissions and hippocampal neurogenesis [19,29,30], whereas the role of spinal LPA1 receptor is not much known. For example, abnormal hippocampal neurogenesis [31] and impaired hippocampus-dependent spatial memory and cognition were observed in the brain LPA1 receptor–deficient adult mice [32]. Considering the LPA1 receptor is also expressed in spinal cords [33], the first mechanism is that gintonin might achieve its effects for attenuation of ALS symptoms through spinal LPA1 receptor regulation. Supporting this notion, the spinal cord reduction of LPA1 receptor protein expression in G93A-SOD1 Tg mice was restored after gintonin treatment (Fig. 6). The second mechanism

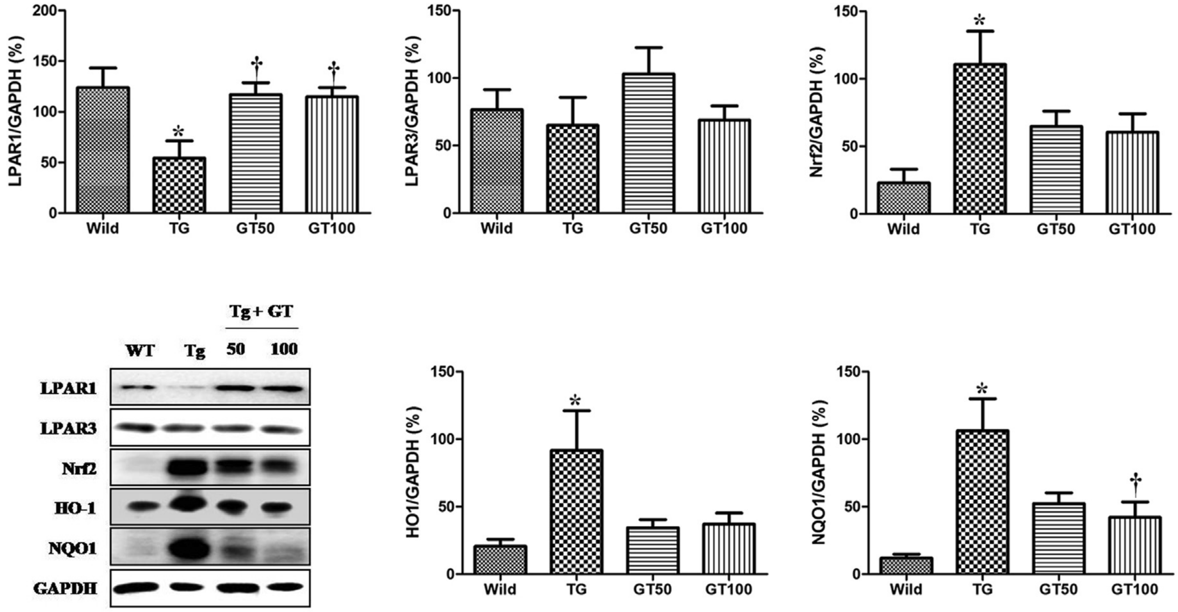

Fig. 6. Effects of gintonin on spinal LPA1/3 receptors and Nrf2/HO1 in G93A-SOD1 TG mice. Immunoblot for LPA1/3 receptors and Nrf2/HO1 in the lumbar spinal cord of wild-type (wild), G93A-SOD1 (TG), G93A-SOD1 + gintonin (50 mg/kg, GT50), and G93A-SOD1 + gintonin (100 mg/kg, GT100) groups (n = 3-5 per group; $^{*}P < 0.05$, indicating a significant difference compared with the wild group; $^{\dagger}P < 0.05$, indicating a significant difference compared with the TG group; $^{\#}P < 0.05$, indicating a significant difference compared with the GT50 group). Data are presented as means ± standard error of the mean.

for the gintonin-mediated attenuation of ALS is that gintonin administration could increase neurotropic factors for the survival of spinal neurons and stimulate neurotransmitter synthesis such as acetylcholine for attenuation of motor dysfunctions. As shown in Fig. 3, oral administration of gintonin increased BDNF and ChAT expressions in spinal cells. In previous studies, *in vitro* or *in vivo* gintonin treatment stimulated the cell proliferation of neuronal precursor cells in addition to brain BDNF and ChAT expression [34,35]. Similarly, gintonin-mediated increases of spinal BDNF and ChAT expression might contribute to the attenuation of neurological symptoms of ALS and prolong the life span in ALS animal models.

The third possibility is that gintonin administration attenuates oxidative stresses associated with spinal ROS production due to mutation of the G93A-SOD1 gene. García-Fernández et al. [36] demonstrated that LPA1 receptor–deficient mice are more susceptible to oxidative stresses, impairment of hippocampal neurogenesis, and subsequent memory dysfunctions. Therefore, the long-term deteriorating effects by the G93A-SOD1 gene mutation might induce spinal reactive oxygen stresses (Fig. 5) resulting in dysfunctions of spinal motor neurons and finally paralysis of motor functions. In our study, we found that the oral administration of gintonin attenuated spinal oxidative stress by reducing oxidative stress-related indicators, including ferritin, iron, and NQO1 (Fig. 5). In our previous report, we reported that gintonin treatment significantly ameliorated pyocyanin-induced reactive oxygen species in a concentration- and time-dependent manner in neuronal culture cells [19]. The previous studies also showed that the attenuation of other herbal medicine-mediated neurological symptoms in the G93A-SOD1 mice models was achieved via reductions of ROS production [21]. These results suggest that gintonin-mediated antioxidative stress activities might help to overcome G93A-SOD1 TG-ROS production and might protect spinal cords from ROS production. However, it is worthwhile to note that although oxidative stress is an important contributor of ALS [37], previous experiments with representative antioxidant agents such as vitamin E and CoQ10 have shown little or no therapeutic effects [38,39]. Therefore, individual treatment of antioxidants might be inadequate for inhibiting the pathological progression of ALS.

Astrocytes and microglia are the main glial cells of the central nervous system, and abnormal activations of these glial cells influence spinal inflammations that regulate the pathological progress in ALS [40]. On this basis, we examined whether gintonin attenuated the increase of Iba1-immunoreactive microglia and S100β-immunoreactive astrocytes in the spinal cords of G93A-SOD1 TG mice. We observed that gintonin can reduce Iba1 expression and astrogliosis by reducing S100β expression (Fig. 4). Therefore, the fourth possibility is that gintonin also showed anti-inflammatory effects by inhibiting Iba1 and S100β expression in spinal microglia, as shown in Fig. 4. Finally, the last possibility is that gintonin-mediated combinational effects (i.e., pleiotropic effects via LPA1 receptor) on spinal neurons, glia, and antioxidants and antiinflammatory effects on the spinal cord might contribute to attenuations of spinal dysfunctions in G93A-SOD1 TG mice.

Interestingly, when ALS-induced regulations of the Nrf2/HO1/NQO1 signaling pathway were examined, increases of spinal Nrf2 protein staining in saline-only treated G93A-SOD1 TG mice and decreases of spinal Nrf2 expression after gintonin administration to G93A-SOD1 TG mice were observed (Fig. 5). In the western blotting study of Nrf2/HO1/NQO1, increases of spinal Nrf2/HO1/NQO1 protein levels in saline-only treated G93A-SOD1 TG mice were observed, whereas gintonin administration decreased spinal Nrf2/HO1/NQO1 protein levels (Fig. 6). Similarly, previous studies have shown that the neuroprotective effects of *Scolopendra subspinipes mutilans* extract and Bojungikgi-tang achieved their effects by reducing the ALS-mediated increase of HO1 in G93A-SOD1 TG mice [22,41,42]. Recently, our *in vivo* study also supports present beneficial effects of gintonin by showing neuroprotection via anti-oxidative stress, antiapoptosis, and antiinflammatory mechanism during brain development [43]. These results indicate that the gintonin-mediated amelioration of ALS could be associated with regulations of oxidative stresses generated during the development of ALS neuropathological and clinical signs.

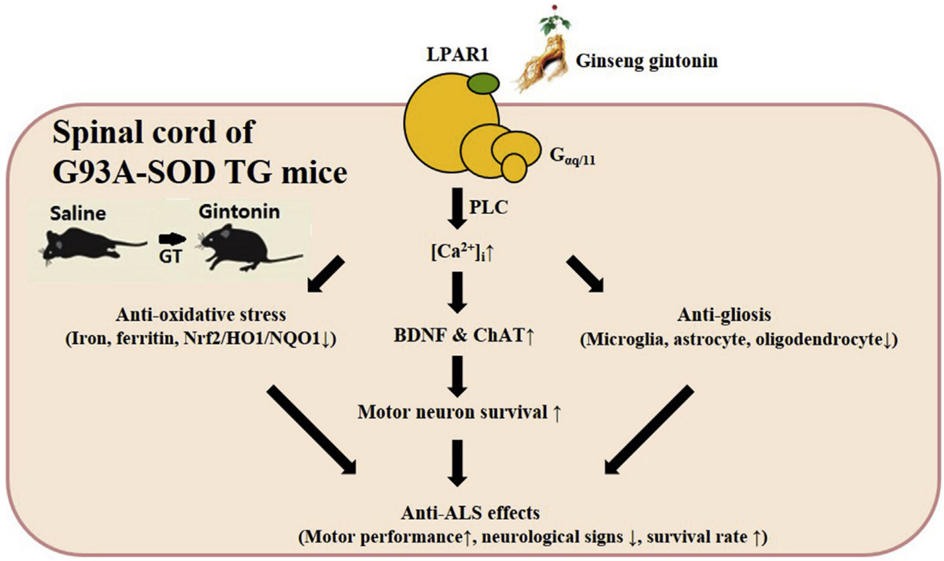

Fig. 7. A schematic illustration of gintonin-mediated anti-ALS effects. Gintonin-mediated anti-ALS effects might include three ways via LPA1 receptor signaling pathways such as antioxidant stress, increases of BDNF and ChAT expressions, and antigliosis. The convergence of three beneficial ways increases the survival of spinal motor neurons and finally survival rates of ALS via enhancement of motor performances and attenuations of neurological symptoms. BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; GT, gintonin; PLC, phospholipase C; ALS, amyotrophic lateral sclerosis.

Our previous studies demonstrated that gintonin exerts anti-neurodegenerative activities. The 14 weeks repeated (three times a week) oral administration of gintonin inhibited amyloid plaque accumulations in the hippocampus and cortex and also enhanced cognitive functions in AD mouse models via the nonamyloidogenic pathway [12]. The long-term administration of gintonin also increased the *in vitro* and *in vivo* hippocampal cholinergic systems in AD mouse models [34]. In addition, in a study using MPTP/MPP^+ animal models of PD, gintonin restored dopaminergic neurons and mitigated behavioral dysfunctions. The molecular basis of the anti-PD action of gintonin is through regulations of the Nrf2/HO-1 signaling, which repress the induction of proinflammatory cytokines, nitric oxide synthase, and apoptosis-related markers in the striatum and substantia nigra of the mice via the LPA1 receptor. In addition, the neuroprotective benefits of gintonin were also displayed by decreasing the accumulation of α-synuclein in the striatum and substantia nigra of PD model mice [14,15].

Moreover, gintonin ameliorated the neurological impairment/lethality and striatal toxicity in cellular or animal models of HD using 3-NPA. The underlying molecular mechanisms also included the alleviation of mitochondrial dysfunctions (i.e. succinate dehydrogenase and MitoSOX activities), microglial activation, mRNA expression of inflammatory mediators (i.e. IL-1β, IL-6, TNF-α, COX-2, and iNOS), and apoptosis in the striatum. Its action mechanism was also associated with the LPA1 receptor and Nrf2 signaling activations and the inhibition of mitogen-activated protein kinases and nuclear factor-κB pathways [16]. In the our study, we further extended that long-term oral administration of gintonin attenuates spinal neurological symptoms and prolongs the survival rate of ALS through gintonin-mediated LPA1 receptor regulations, antioxidant, and antiinflammatory effects (Fig. 7). Taken together, these gintonin-mediated pleiotropic actions might be a molecular basis for the beneficial effects of gintonin against ALS.

5. Conclusion

In summary, we have shown that gintonin can alleviate neurological symptoms and histological changes in G93A-SOD1 mice via multitarget mechanisms, such as antioxidative stress and antiinflammation via the LPA1 receptor, resulting in a prolonged life span. Therefore, gintonin is an essential component of *Panax ginseng* for the amelioration of ALS. Finally, gintonin could be applied for the improvement or amelioration of neurological symptoms related to ALS.

Role of funding source

Nothing declared.

Conflicts of interest

All the authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.04.002.

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