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Bojungikgi-tang Improves Muscle and Spinal Cord Function in an Amyotrophic Lateral Sclerosis Model

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Abstract

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by progressive motor function impairment, dysphagia, and respiratory failure. Owing to the complexity of its pathogenic mechanisms, an effective therapy for ALS is lacking. Herbal medicines with multiple targets have good efficacy and low adverse reactions for the treatment of neurodegenerative diseases. In this study, the effects of Bojungikgi-tang (BJIGT), an herbal medicine with eight component herbs, on muscle and spinal cord function were evaluated in an ALS animal model. Animals were randomly divided into three groups: a non-transgenic group (nTg, $n = 24$), a hSOD1^{G93A} transgenic group (Tg, $n = 24$), and a hSOD1^{G93A} transgenic group in which 8-week-old mice were orally administered BJIGT (1 mg/g) once daily for 6 weeks (Tg+BJIGT, $n = 24$). The effects of BJIGT were evaluated using a rotarod test, foot-printing, and survival analyses based on Kaplan–Meier survival curves. To determine the biological mechanism underlying the effects of BJIGT in hSOD1^{G93A} mice, western blotting, transmission electron microscopy, and Bungarotoxin staining were used. BJIGT improved motor function and extended the survival duration of hSOD1^{G93A} mice. In addition, BJIGT had protective effects, including anti-oxidative and anti-inflammatory effects, in both the spinal cord and muscle of hSOD1^{G93A} mice. Our results demonstrated that BJIGT causes muscle atrophy and the denervation of neuromuscular junctions in the gastrocnemius of hSOD1^{G93A} mice. The components of BJIGT may alleviate the symptoms of ALS via different mechanisms, and accordingly, BJIGT treatment may be an effective therapeutic approach.

Keywords Amyotrophic lateral sclerosis · Bojungikgi-tang · Motor function · Muscle atrophy

Introduction

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease that leads to motor function impairment, dysphagia, and respiratory failure. ALS is a heterogeneous disease and can be divided into two types. Sporadic ALS, with unknown cause, is most frequent. Familial ALS is caused by TAR DNA-binding protein (TAR-DBP), fused in sarcoma (FUS), or a hexanucleotide repeat expansion in an intronic region in chromosome 9 open reading frame 71 (C9ORF72) [1] and in approximately 20% of cases is caused by mutations in the gene encoding copper/zinc superoxide dismutase (SOD1). The mSOD1 animal model mimics the pathogenic progression of human ALS and is widely used for studies of the disease [2].

Preclinical studies using the ALS animal model have demonstrated multiple pathogenic mechanisms, including oxidative stress, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and protein aggregation [3]. The pathogenic mechanisms are highly complex, involving multiple factors, thereby limiting the development of an effective therapy. Riluzole (a glutamate antagonist) and edaravone (an anti-oxidant), which are approved by the Food and Drug Administration (FDA), have been used for ALS patients, but they only extend the survival of patients with ALS by 2–3 months [4]. According to two major hypotheses, muscle atrophy is followed by neuronal cell loss [5] or skeletal muscle is actively involved in the pathogenesis of ALS [6]. Dobrowolny et al. have demonstrated that muscle-specific SOD1^{G93A} expression results in severe muscle atrophy but no motor neuron degeneration [7]. Therefore, drug discovery for ALS should consider multiple targets for the homeostasis of motor neurons and muscle to account for the complexity of the disease.

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Herbal medicines, known as traditional medicines, are commonly used as complementary and alternative medicines. Herbal medicines with multiple targets are beneficial owing to their good efficacy and low adverse reactions for the treatment of neurodegenerative diseases. Recently, the safety and efficacy of herbal medicines have been evaluated for many neurological disorders, including Parkinson's disease, Alzheimer's disease, and dementia [8–10]. Herbal medicines have neuroprotective functions, e.g., against oxidative stress and neuroinflammation, for the prevention and treatment of ALS [11]. However, the precise effects of herbal medicines on ALS and the biological mechanisms underlying these effects are unclear.

Bojungikgi-tang (BJIGT), known as Bu Zhong Yi Qi Tang in Chinese medicine and Hochuekkito in Japan, is composed of eight component herbs (Angelicae Gigantis radix, Astragali radix, *Atractylodis* rhizome, Bupleuri radix, *Cimicifugae* rhizome, Citri unshii pericarpium, Ginseng radix alba, and *Glycyrrhizae* radix); it improves chronic fatigue syndrome and has anti-tumor effects [12, 13]. In addition, BJIGT has been used to improve immune function [14], oncostatic drug-induced leukopenia [15], and allergic rhinitis [16], and to protect against aging [17]. Toshiaki et al. have demonstrated that BJIGT is useful for the treatment of weakness and fatigue in elderly patients [18]. In an animal study, BJIGT elevated the levels of dopamine and noradrenaline in brain tissues and improved learning and memory [17–19]. In addition, the oral administration of BJIGT did not induce toxicity at up to 2000 mg/kg in animals [20]. BJIGT is the fifth most commonly used herbal formula in traditional Korean medicine [21]. Jun et al. reported that BJIGT treatment improves symptoms in patients with ALS patients based on a clinical case study [22]. However, the biological mechanism underlying the beneficial effect of BJIGT on ALS has not been evaluated. In this study, the effects of BJIGT on the function of the muscle and spinal cord were determined using an ALS animal model. The results of this study revealed that BJIGT has protective effects on the motor neurons and muscle in hSOD1^{G93A} mice. Furthermore, our results clarified the molecular mechanism by which BJIGT increases the survival duration in hSOD1^{G93A} mice. Accordingly, BJIGT treatment is a potential therapeutic approach for ALS; different herbal components may function via different mechanisms to alleviate symptoms.

Materials and Methods

Animals

Hemizygous transgenic B6SJL mice carrying a glycine-to-alanine mutation at the 93rd codon of the cytosolic Cu/Zn superoxide dismutase gene (hSOD1^{G93A}) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained as described previously [23]. All mouse experiments were performed in accordance with the US National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees of the Korea Institute of Oriental Medicine (protocol number: 13-109). All animals were allowed access to water and food ad libitum and were maintained under a constant temperature (21 ± 3 °C) and humidity (50 ± 10%) under a 12-h light/dark cycle (with lights on from 07:00 to 19:00).

Bojungikgi-tang Treatment

BJIGT was purchased from Hankookshinyak (Chungnam, Korea) and diluted with autoclaved distilled water. The mice were randomly divided into three groups: a non-transgenic group (nTg, $n = 24$), a hSOD1^{G93A} transgenic group (Tg, $n = 24$), and a hSOD1^{G93A} transgenic group in which 8-week-old mice were orally administered BJIGT (1 mg/g) once daily for 6 weeks (Tg+BJIGT, $n = 24$) (Fig. 1). The dose was based on previous results for adult human subjects (5 g/60 kg body weight/day) [24].

Rotarod Test

Mice were trained every other day for 2 weeks to adapt to the apparatus. After training, motor activity was measured using a rotarod apparatus. Motor coordination was assessed by measuring the time that the mice remained on the rotating rod

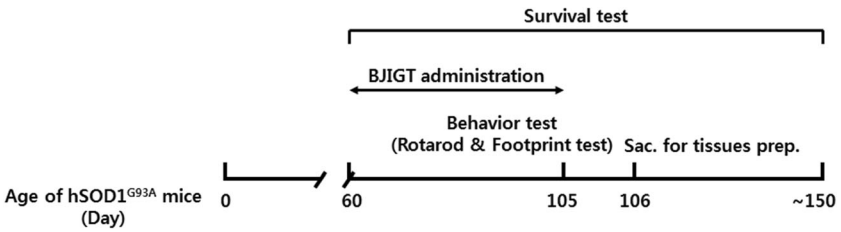

Fig. 1 Timeline of animal experiments. BJIGT (1 mg/g) was orally administered to 60-day-old hSOD1^{G93A} mice. Behavior tests (rotarod and footprint test) were performed until 105 days after 6 weeks of oral administration. For the survival test, BJIGT-treated hSOD1^{G93A} mice were observed until they reached 150 days. Mice were sacrificed for the analysis 6 weeks after BJIGT administration

(10 rpm), as previously described [23]. Three trials were performed for each animal, and the average time spent on the rod was determined for each group.

Footprint Test

The day before mice were sacrificed, a footprint test was performed to measure stride length, which reflects the extent of muscle loosening [25, 26]. The mice crossed an alley of 70 cm in length, 6 cm in width, and 16 cm in height. All mouse hind paws were coated with nontoxic water-soluble ink, and the alley floor was covered with white paper. To obtain clearly visible footprints, at least three trials were conducted.

Life Span Study

For a lifespan analysis, hSOD1^{G93A} mice were assessed for a total of 60 days and divided into the following treatment groups: distilled water-treated SOD1^{G93A} mice ($n = 15$) and SOD1^{G93A} mice treated with BJIGT for 6 weeks ($n = 15$). "Death" was defined according to our previous paper [23]. Survival was analyzed by Kaplan–Meier survival curves using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Tissue Preparation

Animals were anesthetized by an intraperitoneal injection of pentobarbital and perfused with phosphate-buffered saline (PBS). For western blotting, the gastrocnemius muscle (GM) and spinal cord (SP) of transgenic mice were dissected and homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P^{-40}, 0.1% sodium dodecyl sulfate, and 150 mM NaCl) containing protease and phosphatase inhibitor cocktail (Thermo, Waltham, MA, USA). Two tissue homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C and the protein concentration of the lysates was determined using the Bicinchoninic Acid Assay (BCA) Kit (Pierce, Rockford, IL, USA). For histochemical and immunohistochemical analyses, the GM and SP tissues were removed and fixed in 4% paraformaldehyde for 3 days at 4 °C until they were embedded in paraffin. The prepared tissues were sectioned (5 μm thick) and mounted on glass slides. The paraffin sections were de-paraffinized in xylene and rehydrated in a graded alcohol series, followed by dH_2O.

For bungarotoxin staining, GM tissues were embedded in optimal cutting temperature (O.C.T.) compound, frozen in liquid nitrogen, and stored at − 80 °C until sectioning. GM tissues were cut longitudinally at 40 μm thick using a cryostat (Leica, Nussloch, Germany).

Nissl and H&E Staining

Following deparaffinization, the slices were washed in PBS and then stained with 0.1% Cresyl violet (Sigma, St. Louis, MO, USA) for 5 min. Subsequently, slices were dehydrated in a graded series of alcohol (70%, 80%, 90%, and 100%) for 5 min, with two changes, placed in xylene for 5 min, with three changes, and then covered with a coverslip using HistoMount media. For H&E staining, de-paraffinized GM tissues were also washed with PBS, and slices were soaked into hematoxylin solution for 5 min, rinsed in running tap water, stained with eosin for 30 s, and rinsed in water. Finally, slices were dehydrated, a coverslip was added, and the slices were mounted. For the quantification of Nissl staining, the following rules were used: (1) neurons were located in the anterior horn ventral, (2) neurons had a maximum diameter of 20 μm or greater, and (3) neurons had a distinct nucleolus.

Immunohistochemistry

After deparaffinization, the slices were washed in PBS, treated with 3% H_2O_2 to inactivate endogenous peroxidases for 15 min, and washed again in PBS. Sections were then blocked with 5% bovine serum albumin (BSA) in 0.01% PBS-Triton X-100 for 2 h at room temperature. Slices were incubated with the primary antibodies against ionized calcium-binding adaptor molecule 1 (Iba-1) and glial fibrillary acid protein (GFAP) overnight. The next day, the sections were washed with PBS and incubated in the primary matched-secondary antibody for 2 h. For visualization, an ABC solution (Vector Laboratories, Burlingame, CA, USA) was added for 1 h at room temperature and reacted with a 3,3′-diaminobenzidine (DAB) peroxidase substrate solution (Vector Laboratories) within 5 min. Finally, the tissue sections were counterstained with Cresyl violet and a coverslip was added. Immunostained SP sections were observed using a light microscope (Olympus BX51, Tokyo, Japan). For the quantification of immunohistochemical staining, the intensities of Iba-1- and GFAP-positive cells were determined using ImageJ (version 1.46j; NIH, Bethesda, MD, USA).

α-Bungarotoxin Staining

Sliced sections were washed in PBS and then treated with 0.3% Triton X-100 in PBS for 30 min at room temperature. Slices were blocked in 5% BSA/10% goat serum in 0.3% Triton X-100 PBS buffer for 1 h and incubated in the α-bungarotoxin antibody (1:500 dilution) with blocking solution for 2 h. Next, slices were washed in PBS and mounted using anti-fade mounting media.

Western Blotting

GM and SP samples of homogenates (20 μg) denatured with LDS sample buffer (Thermo Fisher Scientific) were separated on Bolt 4–12% Bis-Tris Plus gels (Thermo Fisher Scientific) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). Western blots were blocked in a blocking solution (5% non-fat milk in Tris-buffered saline (TBS) for 2 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The following antibodies were used: tumor necrosis factor (TNF)-α, cluster of differentiation 11b (CD11b), heme oxygenase (HO)-1, ferritin, p62, prospero-related homeobox factor 1 (Prox1), transforming growth factor (TGF)-β, and tubulin (all 1:1000; Abcam, Cambridge, MA, USA); B cell lymphoma-2 (Bcl-2)-associated X protein (Bax), toll-like receptor 4 (TLR4), NAD(P)H quinone dehydrogenase 1 (NQO1), transferrin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); microtubule-associated protein 1A/1B light chain (LC)3B, β-catenin, phosphorylated mechanistic target of rapamycin (pmTOR), mTOR, p-glycogen synthase kinase 3 (pGSK3)β(Ser9), and GSK3β (all 1:1000; Cell Signaling Technology, Danvers, MA, USA); Iba-1 (diluted 1:1000; Wako, Osaka, Japan); and glial fibrillary acidic protein (GFAP, 1:3000; Agilent Technologies, Santa Clara, CA, USA). Subsequently, the membrane was incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and immunoreactivity was visualized using SuperSignal West Femto Substrate Maximum Sensitivity Substrate (Thermo Fisher Scientific). A ChemiDoc Imaging System (Bio-Rad) was used to detect immunoreactive bands; signal intensity was quantified using ImageJ software.

Transmission Electron Microscopy

BJIGT-treated hSOD1^{G93A} mice and age-matched controls were sacrificed and perfused with 2% glutaraldehyde. The procedure for sample preparation for transmission electron microscopy (TEM) was performed as previously described [27]. The sections were observed by using a Tecnai G2 Spirit Twin TEM system (FEI, Hillsboro, OR, USA) and operated at 120 kV.

Statistical Analysis

Data were analyzed using Prism v.5.0 (GraphPad, La Jolla, CA, USA). Results are presented as means ± SEM. Quantitative results were evaluated by one-way analysis of variance followed by the Newman-Keuls post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

BJIGT Improves Clinical Motor Function and Extends Survival in hSOD1^{G93A} Mice

We first evaluated body and muscle weight loss at the symptomatic stage in hSOD1^{G93A} mice after BJIGT administration. As shown in Fig. 2a, body weight was obviously lower in the hSOD1^{G93A} Tg group than in the nTg group ($p < 0.001$). BJIGT treatment for 6 weeks increased the body weight compared to that of mice in the Tg group at the same age, but the difference was not significant. However, the gastrocnemius muscle weight of BJIGT-treated Tg mice was 1.2-fold greater than that of age-matched Tg mice ($p < 0.05$, Fig. 2b–c). To investigate whether the increase in muscle weight by BJIGT affects motor function, we performed behavioral tests, such as rotarod and footprint tests, using nTg, Tg, and BJIGT-treated Tg mice. As shown in Fig. 2d, the motor function of Tg mice was dramatically reduced by 9.2-fold compared to that of nTg mice in the rotarod test ($p < 0.001$). However, BJIGT treatment enhanced motor function by 6.7-fold compared to that of Tg mice ($p < 0.001$). Consistent with these findings, the stride length of Tg mice was 1.4-fold lower (4.4 ± 0.3 cm) than that of nTg mice (6.3 cm ± 0.1 cm) ($p < 0.001$, Fig. 2e). BJIGT treatment significantly prevented the reduction in stride length (6.3 ± 0.2 cm) compared to that of Tg mice (4.4 ± 0.3 cm) ($p < 0.001$, Fig. 2e). Next, we examined whether BJIGT affects the survival of hSOD1^{G93A} mice. As shown in Fig. 2f, BJIGT treatment extended the life span in the ALS animal model harboring the G93A mutation in the human *SOD1* gene. The endpoint was postponed by approximately 138 days ($p = 0.0155$) in BJIGT-treated hSOD1^{G93A} mice compared to the Tg group (129 days) ($p = 0.0155$; Fig. 2f). These findings indicate that BJIGT treatment prevents disease progression and delays the disease endpoint.

BJIGT Attenuates Inflammatory Effects in the Gastrocnemius Muscle and Spinal Cord of hSOD1^{G93A} Transgenic Mice

Inflammatory abnormalities and immune responses are critical factors for motor neuron death and muscle atrophy, contributing to the disease process in ALS. To investigate the effects of BJIGT on inflammation in the gastrocnemius muscle of hSOD1^{G93A} mice, we determined the levels of inflammatory proteins, including the astrocyte marker glial fibrillary acidic protein (GFAP), microglial CD11b, and tumor necrosis factor-alpha (TNF-α). As shown in Fig. 3, the expression levels of GFAP, CD11b, and TNF-α were significantly higher (by 2.6-fold, 6.5-fold, and 4.7-fold) in the

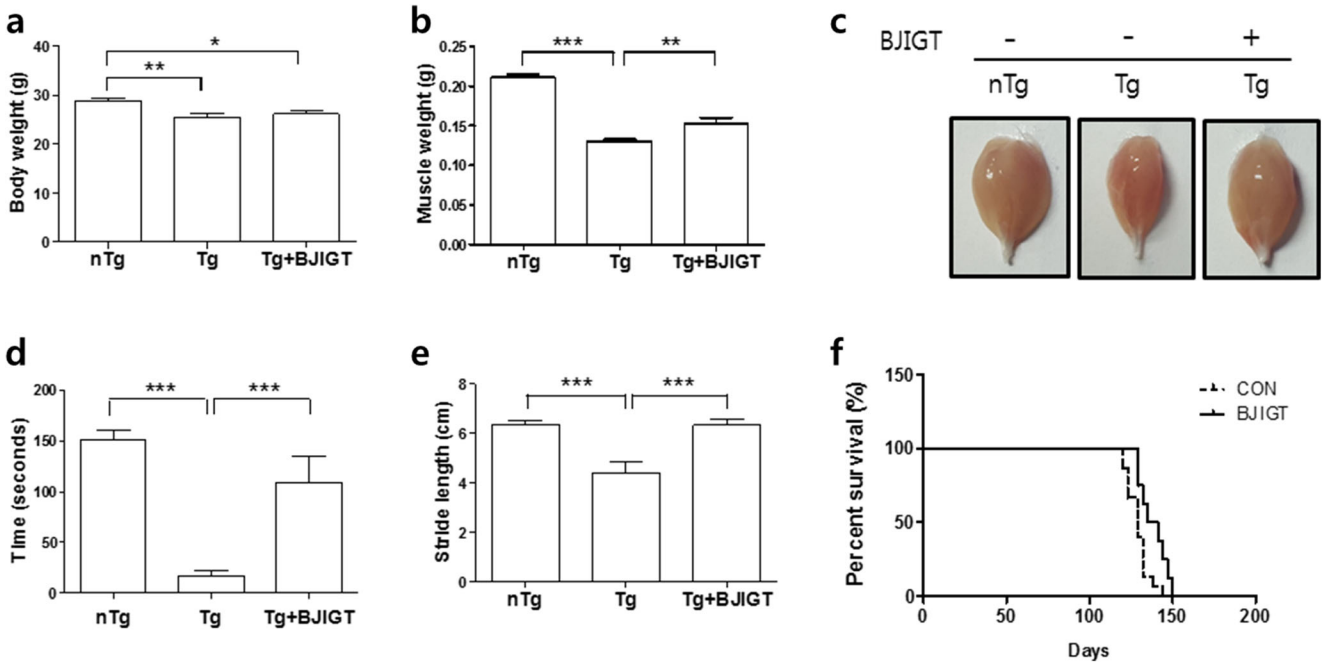

Fig. 2 BJIGT improves motor activity and prolongs life span in hSOD1^{G93A} mice. After the administration of BJIGT for 6 weeks, body weight was measured the day before mice were sacrificed. Body weights of non-transgenic mice, hSOD1^{G93A} mice, and BJIGT-treated hSOD1^{G93A} mice ($n = 9$/group) (**a**), muscle weight (**b**), and images of the gastrocnemius muscle (**c**). Behavioral tests were performed, including the rotarod (**d**) and footprint test (**e**), to determine the effect of BJIGT on motor function in mice. Lifespan after BJIGT treatment was calculated by the Kaplan–Meyer analysis ($n = 15$/group) (**f**). Results are expressed as means ± SEM (**$p < 0.01$, ***$p < 0.001$). nTg: non-transgenic mice, Tg: hSOD1^{G93A} mice, Tg+BJIGT: Bojungikgi-tang-treated hSOD1^{G93A} mice

gastrocnemius muscle of hSOD1^{G93A} transgenic mice than in the muscle of nTg mice (Fig. 3a, b). Furthermore, BJIGT treatment significantly reduced the expression of GFAP, CD11b, and TNF-α by 1.8-fold, 2.4-fold, and 2.8-fold in the gastrocnemius muscle compared to the expression levels in Tg mice, respectively (Fig. 3a, b).

To determine the effect of BJIGT on motor neuron death in the lumber 4–5 spinal cord of hSOD1^{G93A} transgenic mice, we investigated the expression levels of inflammatory proteins by immunoblotting and immunohistochemical staining. We observed that BJIGT treatment significantly reduced the levels of neuroinflammation-related proteins, i.e., GFAP, Iba-1, and TLR4, by 1.8-fold, 2.9-fold, and 2.4-fold in the spinal cord of Tg mice (Fig. 3c, d). These findings were confirmed by the relative immunoreactivities of Iba-1 and GFAP and the extent of positive Nissl staining, an indicator of motor neurons. As shown Fig. 3e, the number of motor neurons decreased by 2.7-fold in the ventral horn of the L4–L5 region of the spinal cord in Tg mice compared to the number observed in nTg mice. However, BJIGT treatment significantly prevented the loss of motor neurons by 2.1-fold in the ventral horn of the L4–L5 region of the spinal cord in Tg mice (Fig. 3e). In addition, Iba-1- and GFAP-positive cells were significantly reduced by 3.6-fold and 2.4-fold in the ventral horn of the L4–L5 region of the spinal cord in BJIGT-treated Tg mice compared to Tg mice (Fig. 3f, g).

BJIGT Has Anti-oxidant Effects in the Gastrocnemius Muscle and Spinal Cord of hSOD1^{G93A} Transgenic Mice

Since oxidative stress is a pathological mechanism related to the motor neuron injury and cytoskeletal dysfunction in ALS [28], we investigated the anti-oxidative effects of BJIGT in the gastrocnemius muscle and spinal cord of hSOD1^{G93A} transgenic mice. As shown in Fig. 4a–b, the levels of oxidative stress-related proteins, including HO-1, ferritin, and NQO1, were 5.8-fold, 12.7-fold, and 5.5-fold higher in the gastrocnemius muscle of Tg mice than in nTg mice. Furthermore, BJIGT treatment significantly reduced the expression levels of HO-1, ferritin, and NQO1 by 2.9-fold, 2.2-fold, and 3.8-fold in the muscle compared to those of the Tg group (Fig. 4a–b).

We also observed that BJIGT treatment increased the anti-oxidative effects in the spinal cord of hSOD1^{G93A} transgenic mice. As shown in Fig. 4c–d, the oxidative stress-related proteins HO-1 and Transferrin were significantly reduced by 3.2- and 3.5-fold in the lumber 4–5 spinal cord of BJIGT-treated Tg mice (Fig. 4c–d). Since oxidative stress leads to mitochondrial dysfunction [29], we investigated the structure of mitochondria in the gastrocnemius muscle and spinal cord of hSOD1^{G93A} transgenic mice. As shown in Fig. 4e, mitochondria were disrupted by broken cristae in the gastrocnemius muscle and spinal cord of Tg mice, but BJIGT treatment inhibited the disruption of cristae. In addition, the expression of pro-apoptotic BAX was significantly increased by 13.5-

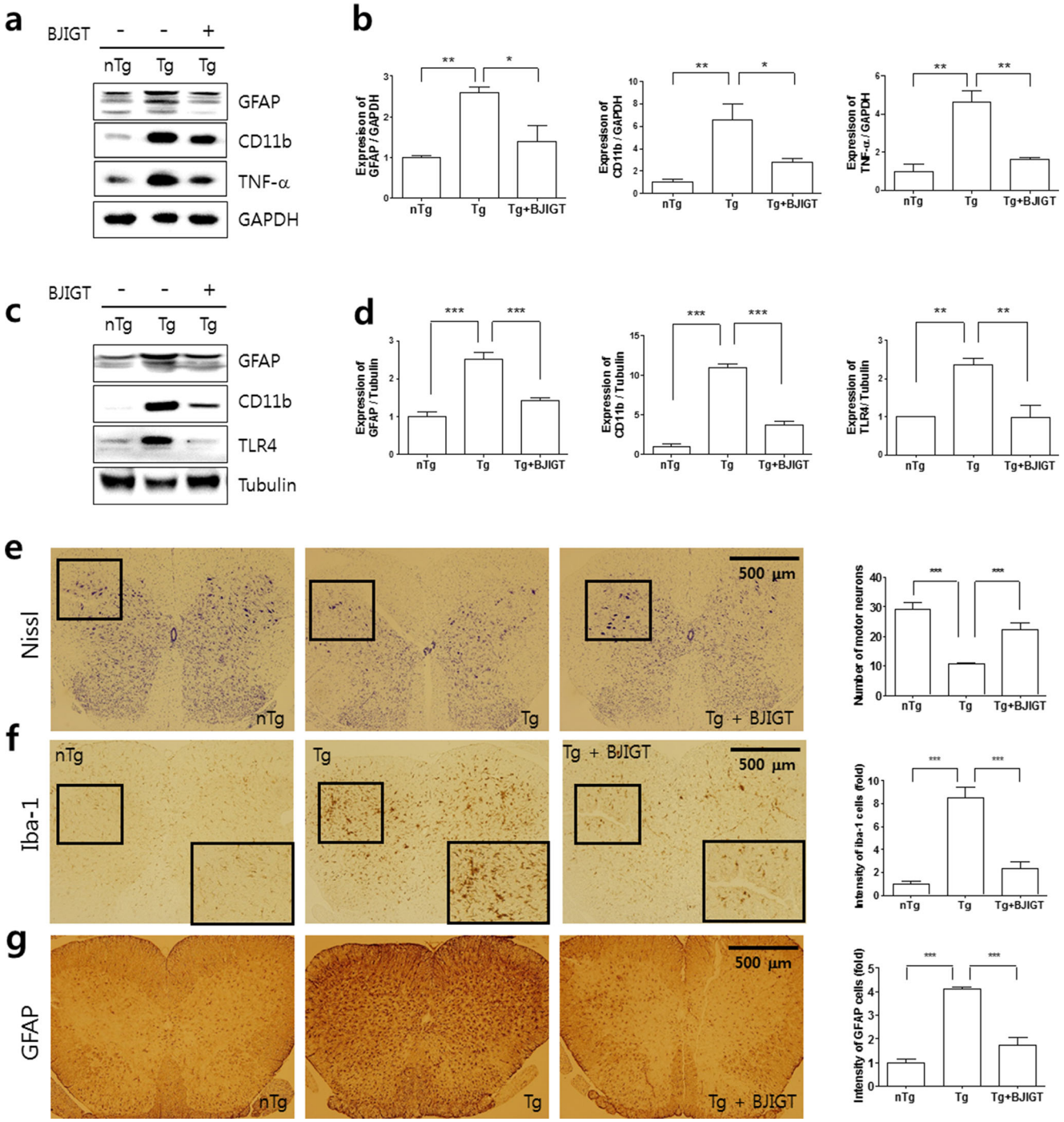

Fig. 3 BJIGT attenuates inflammation in the gastrocnemius muscle and spinal cord in hSOD1^{G93A} mice. The mouse gastrocnemius muscle was immunoblotted with anti-GFAP, anti-CD11b, and TNF-α using nTg, Tg, and BJIGT-treated Tg ($n = 3$/group) (**a**). Protein expression was quantified compared to GAPDH, which was used as a loading control (**b**). Spinal cord (L4–L5) tissue was immunoblotted with anti-GFAP, anti-Iba-1, and TLR4 in nTg, Tg, and BJIGT-treated Tg mice (**c**). Quantification of bands shown in panel (**d**). Tubulin was used as a loading control. Spinal cord (L4–L5) tissue was observed by Nissl staining (**e**) and immunohistochemistry with anti-Iba-1 (f) and anti-GFAP ($n = 6$/group) (**g**). Positive area of Iba-1 and GFAP in each group calculated by ImageJ. Original magnification × 100; bar represents 500 μm. Results are expressed as means ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistical significance was calculated using one-way ANOVA with Newman-Keuls post hoc tests. nTg: non-transgenic mice, Tg: hSOD1^{G93A} mice, Tg+BJIGT: Bojungikgi-tang-treated hSOD1^{G93A} mice

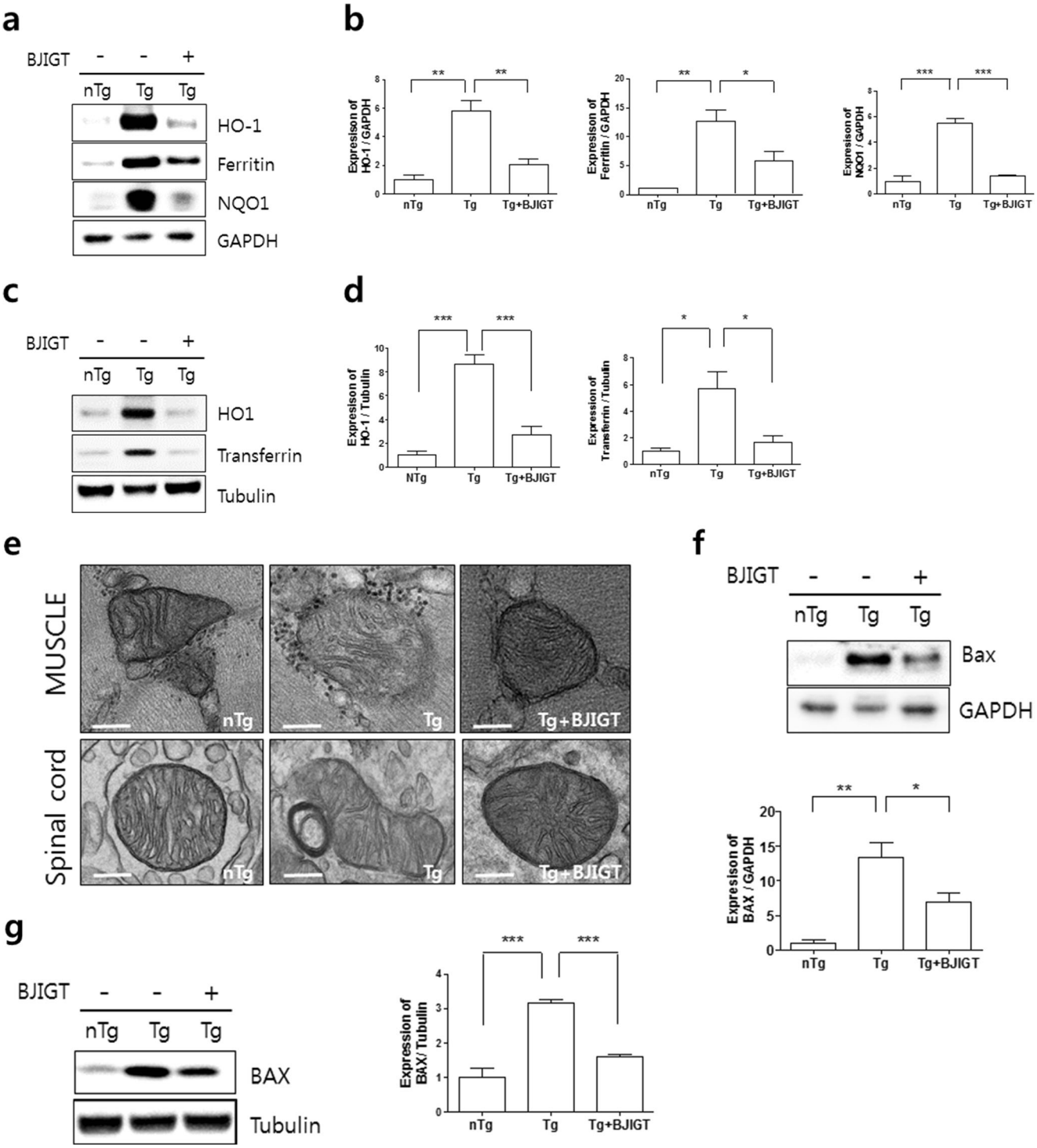

Fig. 4 BJIGT reduces oxidative stress-related proteins in the gastrocnemius muscle and spinal cord in hSOD1^{G93A} mice. Expression levels of oxidative stress-related proteins (HO-1, ferritin, and NQO1) were significantly reduced by BJIGT treatment in the gastrocnemius muscle (**a**) and spinal cord of hSOD1^{G93A} mice ($n = 3$/group) (**c**). Quantification of each protein compared to GAPDH (**b**) or tubulin (**d**). Transmission electron microscopy (TEM) of mitochondria in the gastrocnemius muscle and lumbar spinal cord of distilled water- or BJIGT-treated hSOD1^{G93A} mice (**e**). Bars = 500 nm. The BAX image is representative of three independent experiments in the gastrocnemius muscle (**f**) and spinal cord (**g**) of nTg, Tg, and BJIGT-treated Tg mice ($n = 3$/group). Results are expressed as means ± SEM ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical significance was calculated using one-way ANOVA with Newman-Keuls post hoc test. nTg: non-transgenic mice, Tg: hSOD1^{G93A} mice, Tg+BJIGT: Bojungikgi-tang-treated hSOD1^{G93A} mice

and 3.2-fold in the gastrocnemius muscle and spinal cord of Tg mice, respectively (Fig. 4f–g). However, BJIGT treatment effectively attenuated the expression of BAX by 1.9- and 2-fold in the gastrocnemius muscle and spinal cord of Tg mice, respectively. These results demonstrated that BJIGT has antioxidative effects by preventing mitochondrial disruption in the muscle and spinal cord of the ALS model.

BJIGT Regulates Autophagy Dysfunction in the Gastrocnemius Muscle and Spinal Cord of hSOD1^{G93A} Transgenic Mice

Autophagy is implicated in various neurodegenerative disorders; it ensures the clearance of intracellular aggregates and neuronal survival [30]. Crippa et al. have demonstrated that the well-known autophagic markers LC3B and p62 are much more highly expressed in the muscle than in the spinal cord in ALS mice [31].

To investigate the effect of BJIGT on autophagy dysfunction in the muscle, we examined the expression levels of LC3B and p62 by immunoblotting using the gastrocnemius muscle of Tg mice. As shown in Fig. 5, LC3B and p62 levels were 3.3-fold and 4.5-fold higher in the gastrocnemius muscle of Tg mice than in nTg mice. However, BJIGT significantly decreased the levels of LC3B and p62 by 1.6-fold and 1.8-fold in the gastrocnemius muscle of Tg mice, respectively (Fig. 5a–b).

Transforming growth factor type β (TGFβ) levels are elevated in the serum, plasma, and cerebrospinal fluid in patients with ALS and may induce protein aggregation by autophagy dysfunction [32]. To assess the effect of BJIGT on autophagy dysfunction in the spinal cord of hSOD1^{G93A} transgenic mice, we examined the expression of TGFβ and pmTOR in the spinal cord of hSOD1^{G93A} transgenic mice. Consistent with previous findings, the levels of TGFβ and pmTOR were significantly increased by 3.7- and 2.4-fold in the spinal cord of Tg mice compared to those of nTg mice. However, BJIGT treatment effectively reduced the levels of TGFβ and pmTOR by 2.6- and 2.0-fold in the spinal cord of Tg mice, respectively (Fig. 5c–d). Those findings suggest that BJIGT treatment improves autophagy in both the muscle and spinal cord in hSOD1^{G93A} transgenic mice.

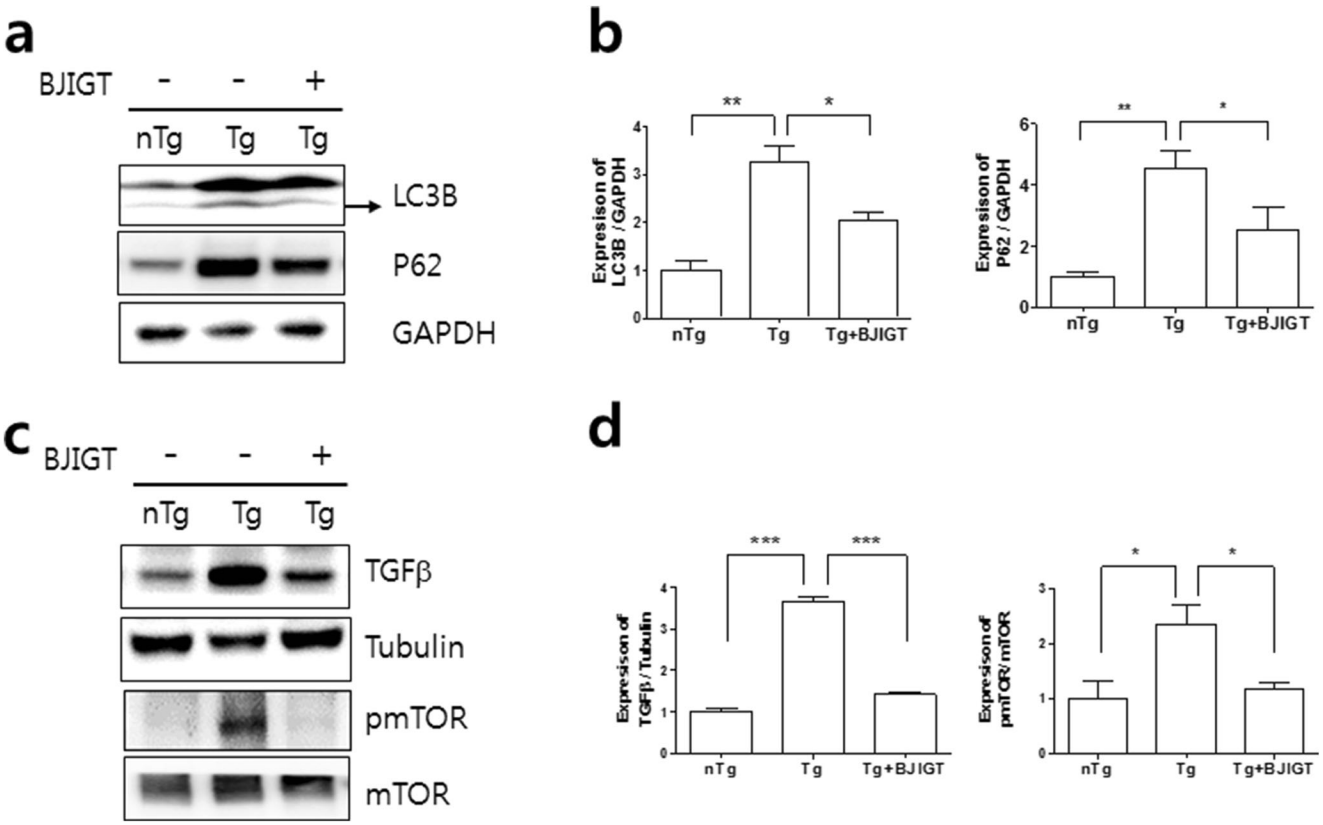

Fig. 5 BJIGT modulates autophagic dysfunction in the gastrocnemius muscle and spinal cord of hSOD1^{G93A} mice. Representative western blots showing the expression of LC3B, p62, and GAPDH (used as the loading control) in the gastrocnemius muscle of hSOD1^{G93A} mice ($n = 3$/group) (**a**). Quantification of each protein normalized to GAPDH (**b**). The expression level of TGFβ and phospho-mTOR in the spinal cord of hSOD1^{G93A} mice ($n = 3$/group) (**c**). Quantification of each protein normalized to tubulin or total mTOR for phospho-mTOR (**d**). Results are expressed as means ± SEM ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Statistical significance was calculated using one-way ANOVA with Newman-Keuls post-hoc test. nTg: non-transgenic mice, Tg: hSOD1^{G93A} mice, Tg+BJIGT: Bojungikgi-tang-treated hSOD1^{G93A} mice

BJIGT Prevents Muscle Atrophy and the Denervation of Neuromuscular Junctions in the Gastrocnemius Muscle of hSOD1^{G93A} Tg Mice

In a previous study, we observed a relatively small muscle fiber diameter and abnormal muscle fiber nuclei in the skeletal muscle of hSOD1^{G93A} transgenic mice [33]. To analyze the alterations in the structure of the gastrocnemius muscle fiber by BJIGT administration, we performed hematoxylin/eosin staining in coronal sections of muscle tissues. At a glance, we found that the fiber diameter was large, similar to that of the muscle of nTg mice, and abnormal nuclei decreased in the gastrocnemius muscle fiber compared to those of the Tg group (Fig. 6a).

In addition, fibrosis in the skeletal muscle results from muscular dystrophies or denervation in ALS. To determine the effect of BJIGT on fibrosis, we examined the expression level of TGFβ and the muscle denervation atrophy-related protein prospero-related homeobox (Prox)1 in the muscle of hSOD1^{G93A} transgenic mice. As shown in Fig. 6b, the levels of TGFβ and Prox1 were dramatically increased by 4.6- and 6.7-fold in the gastrocnemius of Tg mice compared to those of nTg mice. However, BJIGT treatment effectively reduced the expression levels of TGFβ and Prox1 by 3.0- and 1.5-fold in the gastrocnemius of Tg mice, respectively (Fig. 6c).

Since ALS is characterized by the denervation of neuromuscular junctions (NMJs) before the death of motor neurons and the presentation of symptoms [34], we studied on the effect of BJIGT on the denervation of NMJs in hSOD1^{G93A} transgenic mice. As shown in Fig. 6b, the number of α-bungarotoxin (a marker of acetylcholine receptor)-positive cells was significantly lower (by 3.2-fold) in the Tg group mice than in the nTg group. However, BJIGT treatment elevated the number of α-bungarotoxin stained cells by 2.8-fold in the gastrocnemius muscle of mice in the Tg group (Fig. 6d). Wnt proteins are a family of conserved, secreted signaling molecules involved in neuromuscular development and regeneration [35]. Wnt-dependent signaling proteins are highly expressed in the skeletal muscle, at the NMJ, and in motor neurons [36]. In this study, we confirmed that the expression of β-catenin, a wnt signaling protein, was significantly higher (by 6.1-fold) in the gastrocnemius muscle of Tg mice than in nTg mice (Fig. 6e, f). However, BJIGT treatment dramatically reduced β-catenin levels by 1.8-fold in Tg mice. In addition, the expression level of inactive pGSK3β (Ser9) was significantly lower (by 3.1-fold) in the gastrocnemius muscle of Tg mice than in nTg mice, but BJIGT treatment effectively increased inactive pGSK3β levels by 1.8-fold compared to those in the gastrocnemius muscle of Tg mice (Fig. 6e, f). These findings suggest that BJIGT could delay disease progression by the prevention of muscle atrophy and denervation of NMJs and could prevent the loss of muscle-nerve connection in hSOD1^{G93A} Tg mice.

Discussion

For the first time, we characterized the biological mechanism underlying the effects of BJIGT in both the muscle and spinal cord of an ALS animal model. BJIGT treatment improved motor function by reducing inflammation, autophagy dysfunction, and oxidative stress in the muscle and spinal cord of hSOD1^{G93A} Tg mice. Furthermore, we demonstrate that BJIGT treatment extends the survival time and prevents NMJ denervation in the muscle of hSOD1^{G93A} Tg mice.

Inflammatory abnormalities and immune responses are critical factors for motor neuron death and muscle atrophy, contributing to the disease process in ALS. Motor neuron degeneration and cell death involve non-neuronal cells, such as microglia and astrocytes. An increase in astrocytosis and microgliosis by reactive astrocytes and microglia, a clear sign of an immune response, leads to motor neuron degeneration in both the brains and spinal cord of patients with ALS and in animal model of ALS [37]. Inflammation in the skeletal muscle increases the abnormal expression of glial markers, such as glial fibrillary acidic protein (GFAP), p75 neurotrophin receptor, and S100β, leading to ALS pathology [38]. Non-neuronal cells contribute to pro-oxidative and neuroinflammatory events in ALS. Microglia cells produce ROS and recruit inflammasomes, inducing IL-1β and IL18. Pro-inflammatory cytokines (TNF-α and IL-1β) promote neurotoxicity, leading to motor neuron death [39]. Activated astrocytes are also induced by inflammatory cytokines and ROS and then impair glutamate clearance by downregulating GLT1/EAAT2, leading to neurotoxicity [40]. Minocycline, an antibiotic and anti-inflammatory agent, failed in a phase III trial with patients with ALS [41], despite showing neuroprotective and anti-inflammatory effects in an ALS animal model [42]. Therefore, ALS therapeutic strategies should be multi-targeted to cover complex pathological mechanisms. Herbal medicines consisting of multiple compounds could be effective therapies for complex diseases.

In our study, GFAP and CD11b levels were increased in the gastrocnemius muscle and the spinal cord in hSOD1^{G93A} Tg mice, but BJIGT effectively reduced the expression levels of inflammatory proteins. Further analyses, including Nissl staining, showed motor neuronal survival was elevated in BJIGT-treated hSOD1^{G93A} Tg mice. Furthermore, BJIGT treatment improved motor function and extended the life span of hSOD1^{G93A} Tg mice. Taken those results, BJIGT has protective effects on motor neuronal survival and an anti-inflammatory effect in the spinal cord and gastrocnemius muscle of hSOD1^{G93A} Tg mice.

Defects of motor neurons result in muscle dysfunction, leading to progressive paralysis. In the case of ALS, the functional aberrations of the skeletal muscle involve mitochondrial dysfunction [43], oxidative stress [7], and metabolic deficits [44]. In addition, muscle atrophy induced by a

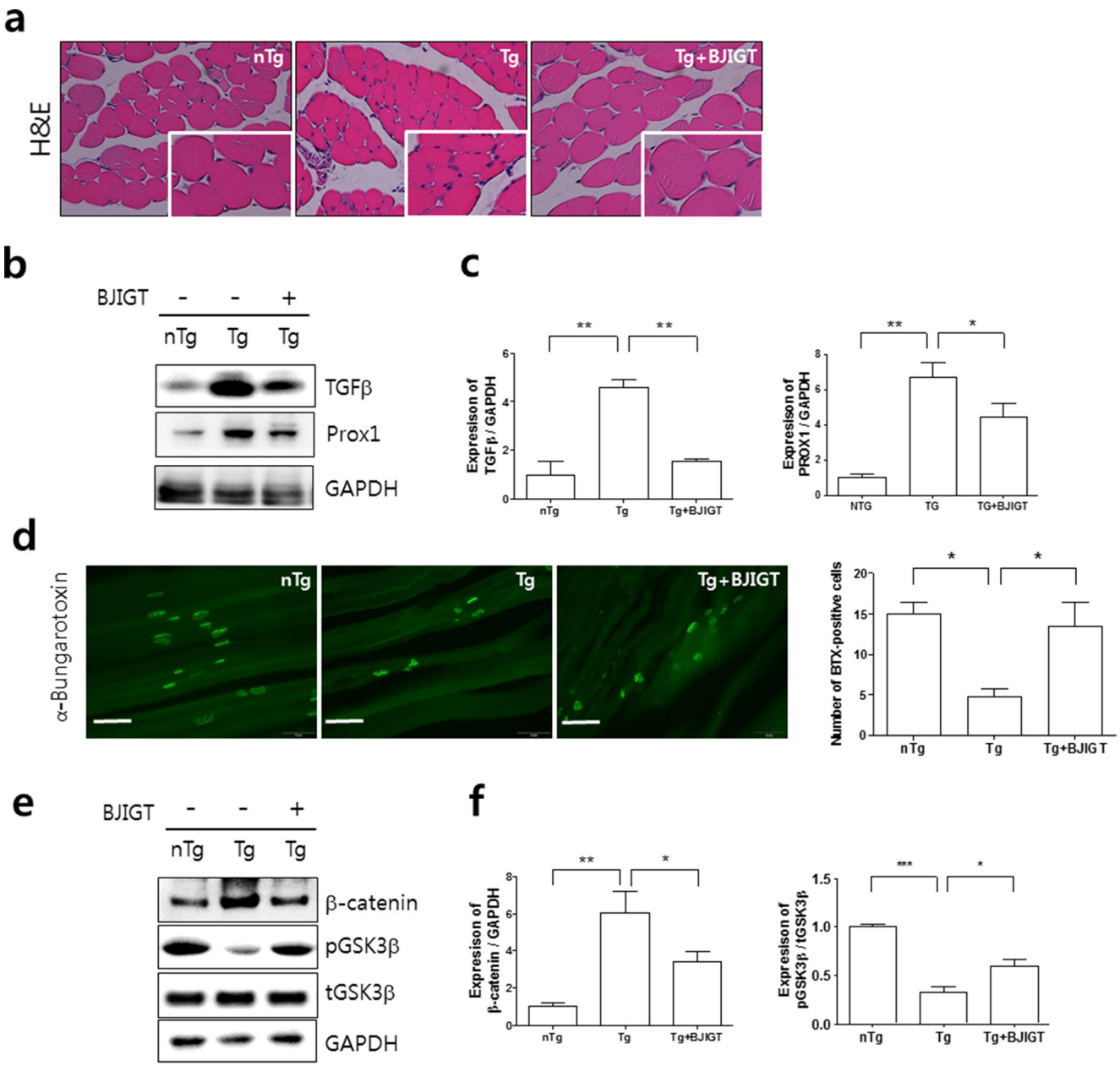

Fig. 6 BJIGT prevents muscle atrophy and mitochondrial dysfunction in the gastrocnemius muscle of hSOD1^{G93A} mice. Muscle morphology and analyses of NMJs in the gastrocnemius muscle of nTg, Tg, and BJIKT-treated Tg mice. The morphology of the muscles was detected by hematoxylin/eosin (H&E) staining (**a**) and NMJs were analyzed by α-bungarotoxin immunostaining (green, **d**). Inserted box is magnified in each H&E stained. TGFβ and prox1 were regulated by BJIGT treatment in the gastrocnemius muscle of hSOD1^{G93A} mice ($n = 3$/group) (**b**). Quantification of each protein compared to GAPDH (**c**). Quantification of NMJs (positive for the α-bungarotoxin antibody) in the gastrocnemius muscle from each group ($n = 4$/group) (**d**). Bars = 500 μm. Representative immunoblots for β-catenin, pGSK3β(Ser9), and tGSK in the gastrocnemius muscle of (**e**). Quantification of immunoblot results for β-catenin normalized to GAPDH and pGSK3β(Ser9) normalized to tGSK3β (**f**). Results are expressed as means ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Statistical significance was calculated by one-way ANOVA with Newman-Keuls post hoc test. nTg: non-transgenic mice, Tg: hSOD1^{G93A} mice, Tg+BJIGT: Bojungikgi-tang-treated hSOD1^{G93A} mice

loss of innervation is associated with an increase in ROS generation [45]. However, motor neuron vulnerability by oxidative stress is controversial, irrespective of whether oxidative stress is a primary or secondary cause of ALS. The results of this study show that oxidative stress-related proteins were significantly increased in the muscle and spinal cord of hSOD1^{G93A} Tg mice, but BJIGT effectively reduced the expression levels of oxidative stress-related proteins. Those findings suggest that BJIGT could regulate mitochondrial dysfunction in an ALS model.

Mitochondrial dysfunction affects the accumulation of oxygen free radicals and bioenergetic defects, leading to

cell death in ALS. Mitochondria in the synapse are critical for neuronal energy and normal function in the central nervous system. In our previous studies, we detected mitochondrial defects in the spinal cord of symptomatic hSOD1^{G93A} mice, leading to motor neuron degeneration [23]. According to Dupus et al., denervation and motor neuron degeneration are caused by muscle mitochondrial uncoupling [46]. In patients with ALS, electron transport chain complexes and citrate synthase levels are reduced in the spinal cord mitochondria [47] and cytochrome c oxidase levels are increased in motor neurons [48]. In addition, mitochondrial abnormalities were detected in the skeletal muscle of patients with ALS [49]. Mitochondrial dysfunction is regulated by the overexpression of peroxisome proliferator activated receptor-gamma coactivator 1 alpha (PGC1α), involved in mitochondrial biogenesis in an ALS animal model [50]. In this study, BJIGT treatment prevented mitochondrial dysfunction and abnormal morphologies in the spinal cord and muscle in symptomatic hSOD1^{G93A} mice. These results suggest that BJIGT is a candidate therapeutic strategy to enhance metabolic regulation in ALS. Although ALS is not defined as a metabolic disease, a therapeutic strategy for the regulation of energy metabolism is important to treat ALS, which is caused by complex pathological mechanisms, including epigenetic mechanisms [51]. Takanashi et al. reported that BJIGT (Hochekkito in Japan) restores metabolic homeostasis, activating mitochondrial and glycolytic energy metabolism [47].

Autophagy is a major protein degradation pathway involved in the clearance of misfolded or aggregated proteins and damaged organelles in both physiological and pathophysiological environments. In addition, autophagy dysfunction is a pathogenic mechanism in numerous neurodegenerative disorders. Muscle atrophy is initiated by the AKT signaling pathway and upregulates the expression of autophagic markers, including LC3B, Bnip3, and cathepsin [52]. Autophagic abnormalities accelerate the denervation of muscle fibers, onset, protein aggregates in interneurons, and glial inflammation [53]. Motor neurons with defects in autophagic flux and lysosomal dysfunction lead to an inflammatory response in hSOD1^{G93A} mice [54]. Although some research has suggested that TGFβ1 could be a pro-survival factor for motor neurons [55, 56], Endo et al. have demonstrated that TGFβ1 released by astrocytes causes motor neuron death [57]. In addition, Phatnani et al. have shown that astrocyte-derived TGFβ1 induces autophagy disruption, leading to protein aggregation via the mTOR pathway under chronic degenerative diseases, such as ALS [58]. Our results confirmed that there is an increase in the expression of autophagic markers, such as LC3B and p62, and phospho-mTOR in the muscles and spinal cords of hSOD1^{G93A} mice. However, BJIGT effectively reduced the levels of autophagy-related proteins in the gastrocnemius muscles and spinal cords of hSOD1^{G93A} mice. These findings suggest that BJIGT could regulate autophagic dysfunction in symptomatic hSOD1^{G93A} mice. Gonzalez et al. have reported that fibrosis in the skeletal muscle induced by TGFβ-dependent signaling is accompanied by an increase in inflammatory responses, including CD11b, IL1β, and TNF-α [59], in an ALS animal model. In addition, Prox1 is enriched in the mouse skeletal muscle and regulates the conversion of slow to fast skeletal muscle fiber function [60].

NMJs, serving as the functional units for skeletal muscles, are large chemical synapses between motor neurons and skeletal muscle fibers. Denervated muscle cells secrete immune molecules (interleukins) and metabolic factors [61]. As the disease progresses, re-innervation of muscle fibers by axons from other motor units fail due to muscle atrophy [62]. Therefore, the energy demand is increased for re-innervation in ALS. mSOD1 leads to NMJ abnormalities and muscle pathology largely due to mitochondrial dysfunction and the activation of the cell death pathway [63]. In addition, GFAP expression is upregulated in the NMJs of familial ALS (SOD1^{G93A} transgenic rats) [64]. These findings suggest that increased inflammation in muscle causes NMJ dysfunction, leading motor neuron death. Wnt proteins are a family of conserved, secreted signaling molecules that play a role in neuromuscular development and regeneration [35]. Wnt-dependent signaling proteins are highly expressed in the skeletal muscle, at the NMJ, and in motor neurons [36]. Glycogen synthase kinase-3 (GSK-3) levels are related to neuronal death in ALS [65] and the phosphorylation of GSK3β results in an increase in cytochrome c release from mitochondria and the activation of caspase-9 and caspase-3, leading to mitochondria dysfunction [66, 67]. In this study, we observed that NMJ dysfunction- and muscle paralysis-related proteins were highly expressed in the gastrocnemius of hSOD1^{G93A} mice. However, treatment with BJIGT had positive effects on the NMJ via β-catenin signaling and the inhibition of muscle atrophy in hSOD1^{G93A} mice. Based on our findings, BJIGT could promote motor neuron survival by both enhancing the expression of anti-apoptotic proteins and suppressing the activity of pro-apoptotic proteins in response to oxidative stress via the PI3K/Akt signaling pathway.

However, owing to the complexity of the components of the Chinese medical formula, it is unclear whether a particular bio-active compound acts alone or whether multiple compounds interact to achieve the effects observed in this study. Future studies may reveal the molecular mechanisms underlying the effects of bio-active compounds in BJIGT.

Authors' Contributions MDC performed western blotting and immunohistochemistry experiments and contributed to writing a part of the manuscript; SHL performed the behavior test (rotarod and foot printing) and administered BJIGT; EJY designed the research, performed TEM, analyzed data, and completed the final proof.

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Compliance with Ethical Standards

All mouse experiments were performed in accordance with the US National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees of the Korea Institute of Oriental Medicine (protocol number: 13-109).

Conflict of Interest The authors declare that they have no conflict of interest.

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