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*Original Research*

# Anti-Inflammatory Effects of *Chaenomeles sinensis* Extract in an ALS Animal Model

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**Abstract**

**Background**: Amyotrophic lateral sclerosis (ALS) is a systemic disease with multiple pathological effects, including neuroinflammation, oxidative stress, autophagy, mitochondrial dysfunction, and endoplasmic reticulum stress. Despite many studies seeking to identify and develop effective therapies, effective ALS treatment has yet to be approved. Hence, patients with ALS ultimately experience muscle atrophy and loss of motor neurons. Herbal medicines have been used to treat numerous diseases by modulating multiple biological processes and exerting pharmacological effects, including anti-inflammatory and antioxidant properties. In particular, *Chaenomeles sinensis* Koehne (CS) exhibits anti-hyperuricemic and nephroprotective effects and is used to treat anaphylaxis, viral infections, and neurodegenerative diseases, such as Alzheimer's disease. This study monitored the effects of CS supplementation on muscle function and motor neurons in hSOD1$^{G93A}$ mice, an established ALS animal model. **Methods**: Body weight measurements and behavioral tests were performed; additionally, western blotting and immunohistochemistry analyses were conducted using the mice gastrocnemius, tibialis anterior, and spinal cord. **Results**: CS augmented anti-inflammatory and antioxidant effects in the muscle and spinal cord of hSOD1$^{G93A}$ mice. Furthermore, CS improved motor function and regulated autophagy in the muscles of the hSOD1$^{G93A}$ mice. **Conclusions**: CS might represent a promising supplement for improving motor function and delaying ALS progression. However, its development for clinical use warrants further investigation.

**Keywords:** amyotrophic lateral sclerosis; anti-inflammation; *Chaenomeles sinensis*; hSOD1$^{G93A}$

## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is induced by motor neuron loss through multiple pathological mechanisms, including neuroinflammation, oxidative stress, autophagy, mitochondrial dysfunction, and endoplasmic reticulum stress, leading to muscle atrophy and severely impaired mobility. ALS is caused by mutations in genes, including superoxide dismutase (*SOD1*), TAR DNA-binding protein, chromosome 9 open reading frame 72 (*C9orf72*), optineurin (*OPTN*), and serine–threonine protein kinase-binding kinase 1 (*TBK1*) [1]. These mutations also contribute to immune dysfunction, affecting microglia, peripheral T lymphocytes, and monocytes. Immune dysfunction in sporadic ALS is associated with disease progression and involves pro-inflammatory macrophages [2]. In an animal study, neuroinflammation induced by activated microglia and astrocytes accelerated disease progression and promoted pathological events in ALS [3]. These anti-neuroinflammatory effects may occur via major histocompatibility complex class I (MHCI) protein upregulation and CD8 T lymphocyte-induced muscle innervation and axonal regrowth enhancement. Furthermore, Song *et al.* [4] (2016) demonstrated that increased microgliosis and astrocytosis reduce MHCI in the spinal motor neurons of mSOD1 mice and patients with ALS while promoting neurotoxicity.

Inflammation and oxidative stress are intricately connected, with loss of redox balance underlying immune dysfunction in many neurodegenerative diseases. In ALS, mitochondrial dysfunction causes oxidative stress, resulting in motor neuron death, muscle dysfunction, and disease progression. However, whether oxidative stress is a trigger or by-product of other pathological events remains unclear. Indeed, several antioxidants have demonstrated therapeutic effects in patients with ALS. Conversely, while some antioxidants, such as coenzyme Q10 and vitamin E, delay disease progression in animal models of ALS [5], they are reportedly ineffective in humans [6]. Moreover, although edaravone—an antioxidant that mitigates oxidative stress through the free radical peroxynitrate—has been approved by the Food and Drug Administration (FDA) for treating ALS [7], its use is limited to certain patients [8].

Oxidative stress is involved in autophagy, while autophagy dysfunction causes reactive oxygen species (ROS) accumulation. Moreover, insoluble protein degradation is impaired in ALS, hence, aggregation of misfolded proteins (e.g., SOD1, OPTN, VCP, TDP-43, ubiquitin-2, and FUS) is a pathological marker of the disease [9]. In addition, the autophagy-associated gene *SQSTM1/p62* is mutated in some ALS cases, accumulating in the lumbar anterior horn of mice with ALS [10]. p62/SQSTM1 is associated with

polyubiquitinated proteins and is degraded by autophagy. Similarly, Song *et al.* [11] revealed that the expression of an autophagy marker (microtubule-associated protein 1 light chain 3 (LC3)-II protein) is increased in the spinal cords of human patients and animal models. Furthermore, Fabbrizio *et al.* [12] demonstrated that activating purinergic P2 receptors (P2X7) controls autophagic flux by regulating LC3b and p62 proteins in SOD1G93A mice, linking anti-neuroinflammatory events with autophagy and suggesting several targets for ALS treatments.

Herbal medicines are made from plant extracts comprising many compounds and elicit multitarget pharmacological actions [13]. These medicines typically have anti-inflammatory and antioxidant effects and have, thus, been used to treat many diseases for millennia in China and other Asian countries [14]. Indeed, several active compounds in a preparation elicit their effects by targeting different macromolecules, each at low and safe concentrations, making herbal medicines useful for the treatment and prevention of different diseases.

*Chaenomeles sinensis* Koehne (CS) has been used to treat anaphylaxis, viral infections, and neurodegenerative diseases (e.g., Alzheimer's disease) and has demonstrated anti-hyperuricemic and nephroprotective effects [15,16]. Kang *et al.* [17] demonstrated the antioxidant and anti-inflammatory effects of the CS extract in benign prostatic hyperplasia, and revealed that CS induces the expression of nuclear factor erythroid-2-related factor 2 while reducing that of pro-inflammatory proteins, including cyclooxygenase-2, inducible nitric oxide synthase, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and interleukin (IL)-2. Collectively, this suggests that CS can help manage chronic diseases through its antioxidant and anti-inflammatory effects [17]. Moreover, Kim *et al.* [18] revealed that multiple bioactive compounds from CS extracts act on various targets, exhibiting anti-neuroinflammatory (reduction in NO levels), neurotrophic, and cytotoxic effects *in vitro*.

Although the FDA recently approved riluzole, an anti-glutamatergic agent for treating ALS, it only extends survival by 2–3 months. Therefore, more effective treatments are required for ALS. In this study, we examined the effects of CS extract treatment on hSOD1$^{G93A}$ mice, an animal model of ALS. We demonstrated that the CS extract exerted anti-neuroinflammatory activity and reduced autophagic dysfunction while improving the motor functions of the affected animals. Our findings suggest that CS is a potential candidate for the treatment of inflammation in neurodegenerative diseases, including ALS.

## 2. Materials and Methods

### *2.1 Animals*

Male hemizygous B6SJ/hSOD1$^{G93A}$ mice (8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in an air-conditioned room at 20–23 °C under a 12 h light/dark cycle and relative humidity of 50 $\pm$ 10%. Male hSOD1$^{G93A}$ mice were randomly divided into the following groups: non-transgenic mice (nTg) (n = 8), hSOD1$^{G93A}$ transgenic mice (Tg) (n = 8), and CS extract-treated hSOD1$^{G93A}$ transgenic mice (Tg+CS) (n = 8). The CS extract (1 mg/g) was orally administered to hSOD1$^{G93A}$ transgenic mice once daily for six weeks, starting at eight weeks of age. The nTg and Tg mice were administered distilled water as controls.

### *2.2 Preparation of Chaenomeles sinensis Koehne Extract*

CS was purchased from Kwang Myung Dang Medical Herbs Co. Ltd. (Ulsan, Republic of Korea). CS extract was prepared as previously described [19]. CS was extracted using distilled water. The CS extracts were dried and stored in powder form. Before administration, powdered CS extract was dissolved in distilled water. Human-equivalent doses of CS in mice were calculated based on results from adult human subjects (5 g/60 kg body weight/day) [20].

### *2.3 Measurement of Muscle Weight and Footprint Test*

Mice were euthanized by intraperitoneal injection of avertin (250 mg/kg). Gastrocnemius (GC) muscle weight was measured using a previously described method [21]. The footprint test was performed one day before the mice were sacrificed to prepare tissues for biochemical analysis. The footprint test was conducted as previously described [19]. Stride length was measured by staining mouse hind paws and allowing them to walk on an alley floor (70 cm $\times$ 6 cm $\times$ 16 cm) covered with white paper. Each mouse performed the footprint test thrice; the average stride length was determined for each group.

### *2.4 Western Blotting*

The tibialis anterior (TA) in the anterior leg, GC in the posterior leg, and spinal cord (SP) of each animal were collected for western blot analysis. Tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer and centrifuged. The supernatant was collected, and the total protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Total protein (20 mg) was resolved using a 4–12% SDS-PAGE precast gel (Thermo Fisher Scientific, Cleveland, OH, USA) and transferred onto polyvinylidene fluoride membranes. The blots were incubated with the following specific antibodies: anti-P62 (Cell Signaling Technology, Beverly, MA, USA), anti-LC3b (Cell Signaling Technology), anti-glial fibrillary acidic protein (GFAP) (Agilent Technologies, Santa Clara, CA, USA), anti-CD11b (Abcam, Cambridge, MA, USA), anti-Bax (Santa Cruz Biotechnology, Dallas, TX, USA), anti-HO1 (Abcam), anti-ferritin (Abcam), anti-transferrin (Santa Cruz Biotechnology), anti-SMAD2 (Cell Signaling Technology), anti-$\beta$-actin (Santa Cruz Biotechnology), and Tubulin (Abcam). The blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibodies and developed using

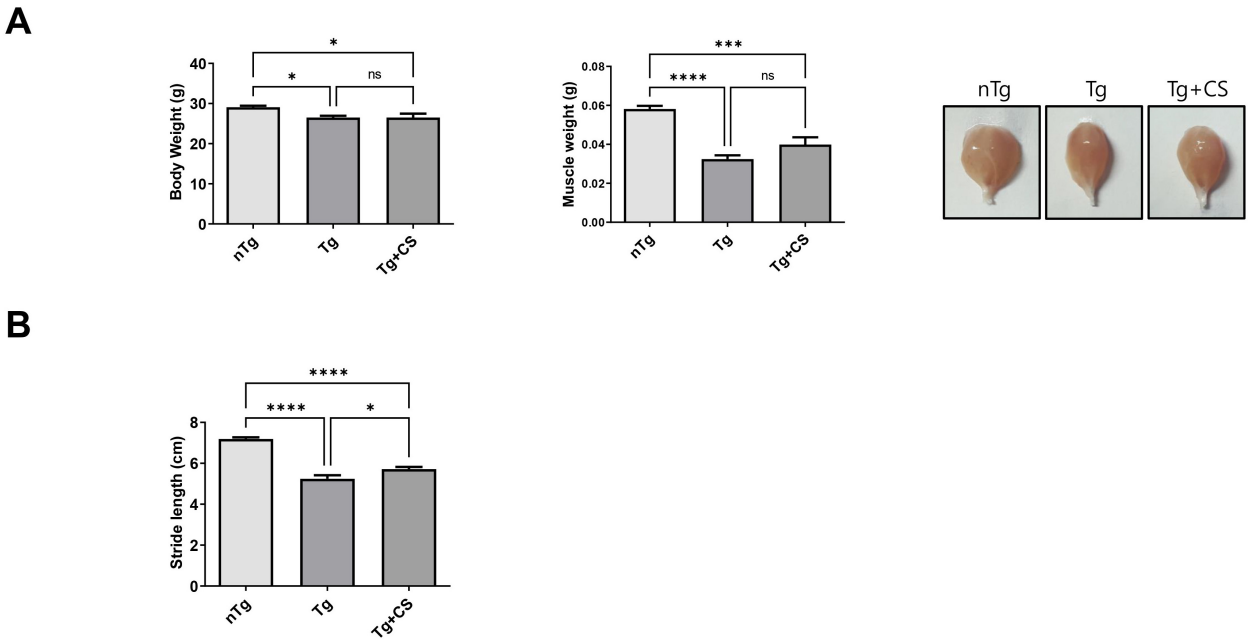


**Fig. 1. *Chaenomeles sinensis* Koehne (CS) improves muscle function in hSOD1$^{G93A}$ mice.** (A) After administration of CS (1 mg/g) for six weeks to hSOD1$^{G93A}$ mice, the body and muscle weight of the gastrocnemius in the posterior leg were measured in the non-Tg, Tg, and Tg+CS groups (n = 6/group). The weight of the gastrocnemius muscle was measured after the skin had been peeled off. (B) To examine motor function, a footprint test was performed in each group of mice (n = 7/group). Bars represent the mean ± tandard error of the mean (SEM). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant; non-Tg, D.W.-treated non-transgenic mice; Tg, D.W.-treated transgenic mice; Tg+CS, *C. sinensis* Koehne-treated transgenic mice.

enhanced chemiluminescence reagents (Thermo Fisher Scientific). The Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) was used for chemiluminescence detection, and immunoblots were quantified using the ImageJ software (v1.43, National Institutes of Health, Bethesda, MD, USA).

### *2.5 Immunohistochemistry*

Spinal cord tissues obtained after sacrificing mice were fixed in 4% paraformaldehyde, embedded in paraffin (Merck), and sectioned. The sections were deparaffinized and incubated with 3% hydrogen peroxide ($H_2O_2$) and 5% bovine serum albumin in 0.01% PBS-Triton X–100 (Sigma-Aldrich, Oakville, ON, Canada). Immunohistochemical staining of SP tissue sections was performed using anti-ChAT (Thermo Scientific, Wilmington, DE, USA), anti-Iba1 (Fujifilm Wako Chemical, Richmond, VA, USA), and anti-GFAP (Agilent Technologies, Santa Clara, CA, USA). Anti-horseradish peroxidase-conjugated mouse or rabbit IgG (GenDEPOT, Katy, TX, USA) secondary antibodies were the added. For visualization, the slides were incubated with diaminobenzidine (DAB) (Vector Laboratories, Newark, CA, USA) before images were captured using an Olympus BX51 microscope (Olympus, Tokyo, Japan), and the intensity of the primary antibodies was measured using the ImageJ software.

Nissl staining was performed using 0.1% cresyl violet. The stained slides were dehydrated using an alcohol gradient and mounted with histomounting media (Sigma-Aldrich, Oakville, ON, Canada).

### *2.6 Statistical Analysis*

Values were presented as mean ± standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons using GraphPad Prism 9 software (GraphPad, Inc., La Jolla, CA, USA). Statistical significance was set at $p < 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.

### *2.7 Ethics Approval*

The Institutional Animal Care Committee of the Korea Institute of Oriental Medicine approved the experimental protocol (korea institute of oriental medicine (KIOM) protocol # 17-061), and all experiments were performed in accordance with the U.S. National Institutes of Health guidelines and guidelines of the Animal Care and Use Committee at the KIOM.

## 3. Results

### *3.1 Chaenomeles sinensis Koehne Improves Motor Activity of hSOD1$^{G93A}$ Mice*

To investigate the effects of CS administration in an ALS murine model, we measured the body and muscle weights and performed a footprint test. The body and

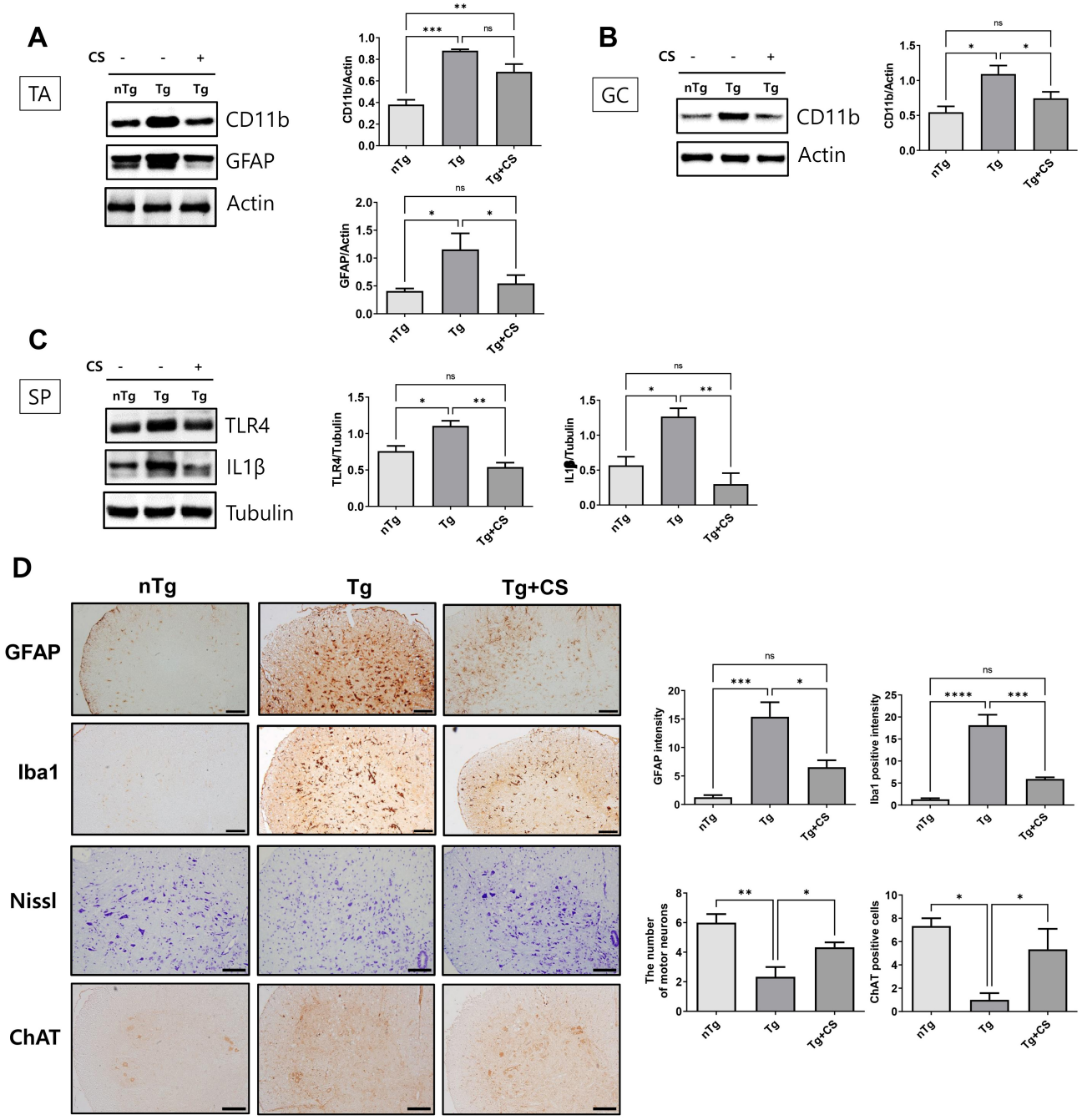


**Fig. 2. *Chaenomeles sinensis* Koehne (CS) attenuates inflammatory events in the muscle and spinal cord of hSOD1$^{G93A}$ mice.** (A) Mice were sacrificed under avertin (250 mg/kg) anesthesia, and the tibialis anterior (TA) in the anterior leg, gastrocnemius (GC) in the posterior leg, and spinal cord (SP) of each animal were collected for western blotting. Western blotting was performed using anti-CD11b and anti-GFAP antibodies in the TA of the non-Tg, Tg, and Tg+CS groups. (B) Immunoblotting with anti-CD11b in GC was performed for the non-Tg, Tg, and Tg+CS groups. Actin was used for the normalization of the immunoblots. (C) Anti-TLR4 and anti-IL-1$\beta$ antibodies were used for western blotting of the spinal cords of the non-Tg, Tg, and Tg+CS groups. Tubulin was used as the loading control. (n = 3, 4/group). (D) Spinal cord tissues obtained after sacrificing the mice were fixed and sectioned for immunohistochemistry. Immunohistochemistry was performed using anti-GFAP and anti-Iba1, along with anti-ChAT and Nissl staining in the spinal cord of the non-Tg, Tg, and Tg+CS groups. Quantification of GFAP and Iba1 intensity was performed using ImageJ software. Scale bar = 100 mm; quantification bars represent the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns, not significant; non-Tg, D.W.-treated non-transgenic mice; Tg, D.W.-treated transgenic mice; Tg+CS, *C. sinensis* Koehne-treated transgenic mice.

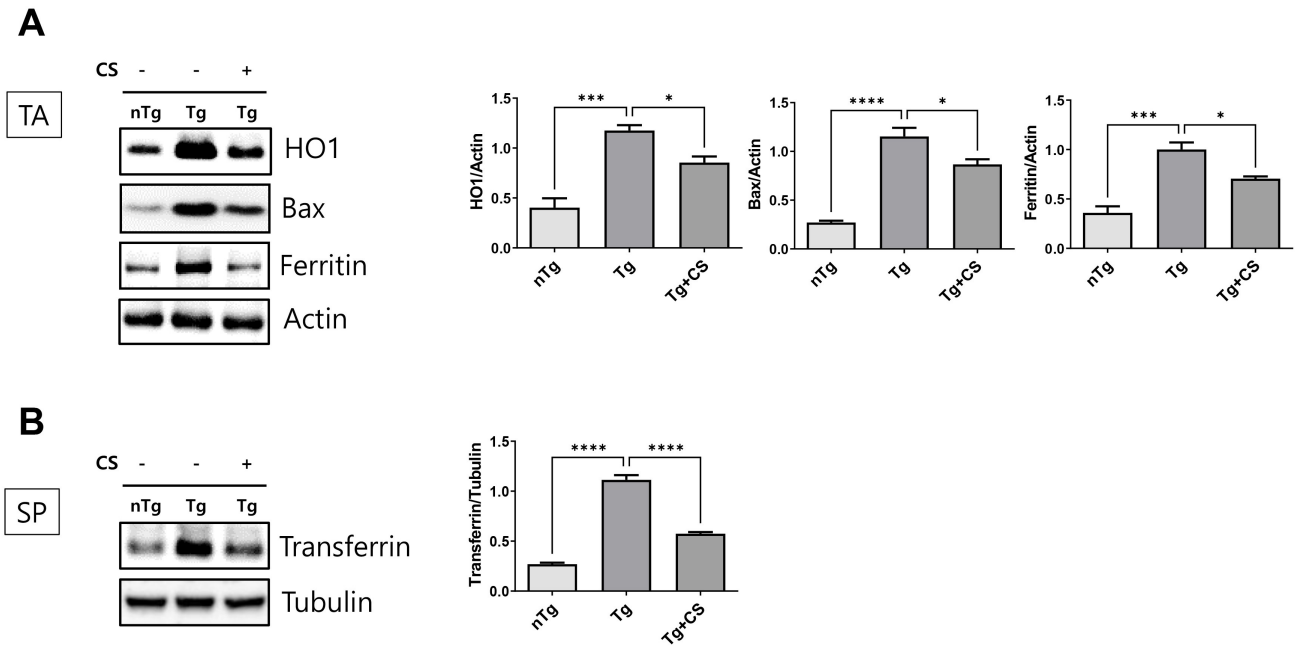


**Fig. 3. *Chaenomeles sinensis* Koehne augments antioxidant activity in the muscle and spinal cord of hSOD1$^{G93A}$ mice.** (A) Immunoblotting was performed with anti-HO1, anti-Bax, and anti-ferritin antibodies in the TA of the non-Tg, Tg, and Tg+CS groups. Actin was used as the loading control. (B) Immunoblots of anti-transferrin in the spinal cord. Tubulin was used to normalize immunoblots (n = 3–4/group). Quantification of immunoblots is represented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. non-Tg, D.W.-treated non-transgenic mice; Tg, D.W.-treated transgenic mice; Tg+CS, *C. sinensis* Koehne-treated transgenic mice.

GC muscle weights of Tg mice were lower than those of nTg mice; however, CS treatment did not alter the body weight of Tg mice (Fig. 1A). CS treatment increased the weight of the GC muscle compared to that in Tg mice; however, these changes were not significant. Furthermore, CS treatment increased stride length by 1.2-fold in Tg mice (Fig. 1B). These results suggest that CS supplementation improves motor activity and muscle function in an ALS murine model.

### *3.2 Chaenomeles sinensis Koehne Attenuates Inflammation in Muscle and Spinal Cords of hSOD1$^{G93A}$ Mice*

To examine the molecular mechanisms underlying the effects of CS on muscle function, we studied its anti-inflammatory effects in the muscles and spinal cords of hSOD1$^{G93A}$ mice. The levels of the inflammation-related proteins CD11b and GFAP were reduced by 1.2- and 2.1-fold, respectively, in the TA of Tg+CS mice compared to the TA of Tg mice (Fig. 2A). In addition, the expression of CD11b was decreased by 1.4-fold in the GC of CS-treated Tg mice compared to the GC of Tg mice (Fig. 2B). CS treatment reduced the expression levels of TLR4 and IL-1$\beta$ by 2.0- and 4.2-fold, respectively, in the SP of CS-treated Tg mice compared to the SP of Tg mice (Fig. 2C). We confirmed the anti-neuroinflammatory effects of anti-GFAP and anti-Iba1 in the SP via immunohistochemical analysis in each group (nTg, Tg, and Tg+CS groups). GFAP- and Iba1-positive intensities were significantly decreased by 2.3- and 3.0-fold, respectively, in the spinal cords of the Tg+CS group compared with those of the Tg group. Furthermore, the abundance of Nissl- and ChAT-positive motor neurons had increased by 2.0- and 5.3-fold, respectively, in the Tg+CS group compared with the Tg group (Fig. 2D). These findings suggest that CS supplementation prevents motor neuron cell death by inhibiting neuroinflammatory signaling in hSOD1$^{G93A}$ mice.

### *3.3 Chaenomeles sinensis Koehne Shows Antioxidant Effects in the Muscles of hSOD1$^{G93A}$ Mice*

Considering the primary role of oxidative stress in inflammatory events, the antioxidant effect of CS was investigated in the muscles and spinal cords of hSOD1$^{G93A}$ mice. In the TA, the levels of the oxidative stress-related proteins HO1, Bax, and ferritin were increased by 2.9-, 4.2-, and 2.7-fold, respectively, in Tg mice compared with nTg mice. However, CS treatment significantly reduced the expression of HO1, Bax, and ferritin by 1.3-, 1.3-, and 1.4-fold, respectively, in the TA of hSOD1$^{G93A}$ mice (Fig. 3A). Similarly, CS treatment reduced the levels of oxidative stress-related proteins (transferrin) by 1.9-fold in the SP of hSOD1$^{G93A}$ mice (Fig. 3B). These results are consistent with the antioxidant effects of CS, which induced improvements in motor function and motor neuron survival in an ALS murine model.

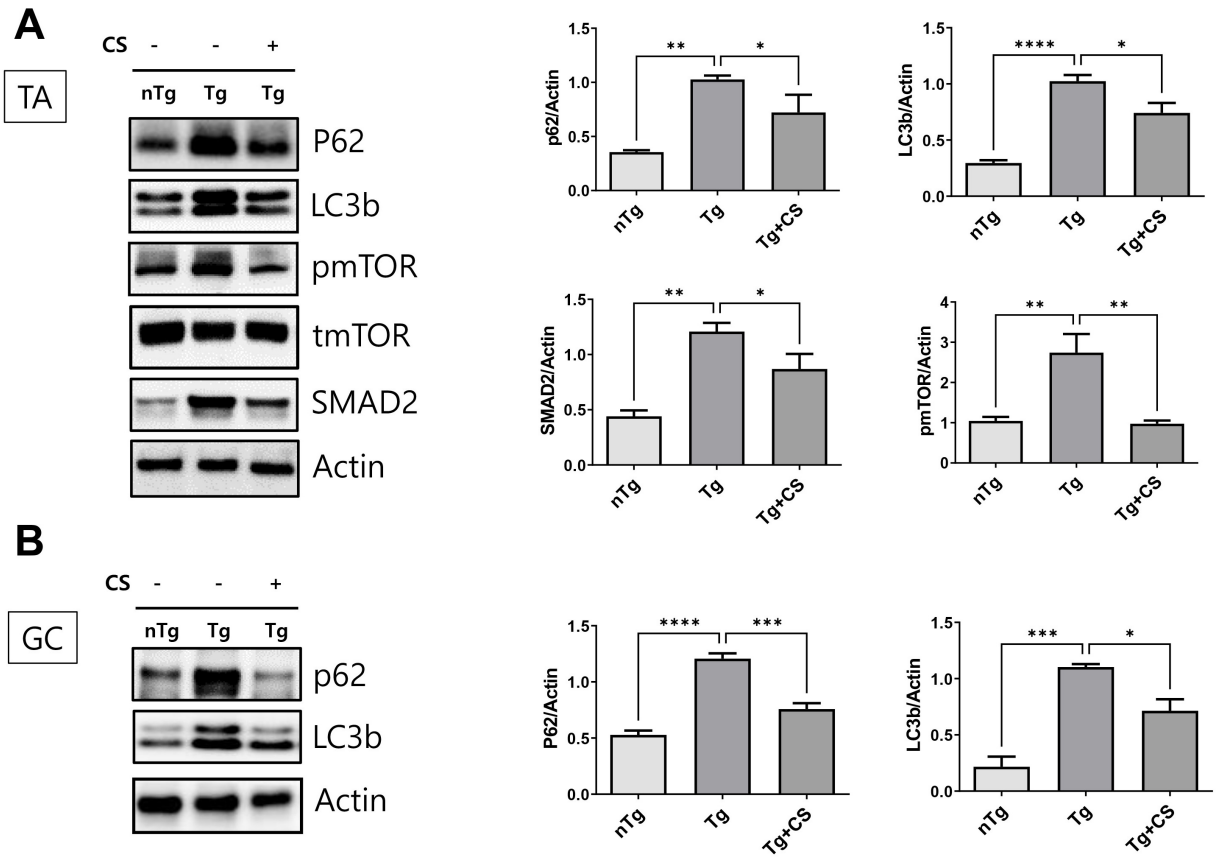


**Fig. 4. *Chaenomeles sinensis* Koehne regulates autophagy dysfunction in the TA and GC of hSOD1$^{G93A}$ mice.** (A) TA and GC tissues from hSOD1$^{G93A}$ mice were prepared for western blot analysis. Autophagy dysfunction-related proteins (p62 and LC3b) and muscle atrophy-related proteins (p-mTOR and SMAD2) were detected using western blotting in the TA of non-Tg, Tg, and Tg+CS groups. (B) Anti-p62 and anti-LC3b antibodies were used for immunoblotting in the GC of the non-Tg, Tg, and Tg+CS groups. Actin was used as the loading control. n = 3–4/group. Quantification of immunoblots is represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. non-Tg, D.W.-treated non-transgenic mice; Tg, D.W.-treated transgenic mice; Tg+CS, *C. sinensis* Koehne-treated transgenic mice.

### *3.4 Chaenomeles sinensis Koehne Regulates Autophagy in the Muscles of hSOD1$^{G93A}$ Mice*

Impaired autophagy can induce muscle dysfunction [22]. Autophagy-related proteins, including p62 and LC3b, were significantly reduced by 1.4- and 1.3-fold, respectively, in the TA of CS-treated hSOD1$^{G93A}$ mice compared with that in hSOD1$^{G93A}$ mice (Fig. 4A). In addition, CS treatment decreased the levels of the muscle atrophy-related proteins p-mTOR and SMAD2 by 1.3- and 2.8-fold, respectively, in the TA of hSOD1$^{G93A}$ mice (Fig. 4A). The p62 and LC3b levels were also reduced by 1.5- and 1.5-fold, respectively, in the GC of CS-treated hSOD1$^{G93A}$ mice compared to those of hSOD1$^{G93A}$ mice (Fig. 4B). These findings suggest that CS prevents muscle atrophy by regulating autophagic dysfunction in hSOD1$^{G93A}$ mice.

## 4. Discussion

ALS is characterized by progressive motor neuron death and muscle atrophy, leading to respiratory failure and death. Although edaravone and riluzole are approved for treating ALS, these medications have limited effects on improving the lifespan, necessitating the development of more effective therapeutic strategies. Therefore, we investigated the effects of CS, an herbal medicine, on the muscles and spinal cords of hSOD1$^{G93A}$ mice in a murine model of ALS. ALS is a systemic disease, and herbal medicines contain various compounds that act on multiple targets. We revealed that the anti-inflammatory and antioxidant effects of CS in hSOD1$^{G93A}$ mice were associated with improved motor activity and muscle function.

Neuroinflammation is a common pathological mechanism of many neurodegenerative diseases. In ALS, neuroinflammation is characterized by increased activation of microglial and astroglial cells. In addition, pro-inflammatory cytokines, such as IL-6, IL-1$\beta$, IL-6, IL-8, vascular endothelial growth factor, and TNF-$\alpha$, have been detected in the peripheral blood of patients with ALS [23]. Activated microglia and astrocytes induce neuroinflammation, which is aggravated by disease progression in the

spinal cords of hSOD1$^{G93A}$ mice [24]. However, given that our study did not determine which specific cytokines are related to ALS, additional investigation on the relationship between cytokine levels and disease severity is warranted to potentially identify new diagnostic and prognostic biomarkers.

Beers *et al.* [24] revealed that CD4+ T lymphocytes augment survival rates by modulating resident microglia and increasing IGF-1 secretion, as T lymphocytes promote protective microglial responses. Activated astrocytes disrupt the clearance of extra glutamate from synaptic clefts due to the loss of the glutamate transporter EAAT2/GLT-1, consequently leading to exacerbated motor neuron degeneration in patients with ALS [25]. Papadeas *et al.* [25] demonstrated that glial-restricted precursor cells transplanted into hSOD1$^{G93A}$ mice differentiated exclusively into astrocytes and facilitated motor neuron death and respiratory dysfunction, a feature of ALS with astrocytic changes. In addition, T cells and CD4+ cells are involved in neuroinflammation in ALS, and abnormal T-cell activation and differentiation in TANK-binding kinase 1 (TBK1) knockout mice augment neuroinflammation via an increase in Foxo 1 phosphorylation [26].

Herbal medicines have been used to prevent diseases worldwide for centuries and are notable in the histories of China, Japan, and Korea. Herbal medicines are extracts and mixtures of multiple bioactive molecules that modulate multiple molecular targets. Some studies have shown that this could contribute to the effectiveness and low toxicity of many herbal medicines. Ginseng is an example of a multitarget herbal medicine. Two disease-modulating compounds with different molecular targets have been isolated from the ginseng plant. Subsequent treatment of an MPTP-based mouse model of Parkinson's disease with ginsenoside Rb1 ameliorated motor deficits and reduced excitotoxicity. This was likely caused by increased expression of the glutamate transporter GLT-1, promoting glutamate uptake in the midbrains and prefrontal cortices of diseased mice [16]. Meanwhile, administration of ginsenoside Rd increased extracellular glutamate clearance via upregulating an astrocytic glutamate transporter via the PI3K/AKT and ERK1/2 pathways [27]. These studies established that an active compound originating from herbal medicines could modulate glutamate systems in treating neuronal diseases caused by glutamate excitotoxicity, such as ALS. Bioactive compounds have been utilized as starting points in medicinal chemistry efforts, guided by network pharmacology and molecular docking analyses [28]. Similar efforts could be applied to bioactive compounds isolated from the CS extract in future work, potentially leading to the identification of compounds with improved properties.

Disease progression in our ALS model (hSOD1$^{G93A}$ mice) was characterized by muscular dysfunction involving impairment of neuromuscular junctions (NMJs) and oxidative stress-induced dysfunction of mitochondria [29]. These dysfunctions are consistent with those observed in other animal models of ALS and human diseases. For example, Xiao *et al.* [22] reported the emergence of elevated cyclophilin D levels in the muscles of patients with advanced ALS, consistent with mitochondrial dysfunction and ROS production. However, further studies are required to elucidate the signaling pathways involved in this process. Graber *et al.* [30] demonstrated that activated macrophages in the peripheral sciatic nerve and increased inflammatory events are associated with motor weakness. Moreover, Dupuis *et al.* [31] reported the involvement of Nogo-A—a member of the reticulon 4 (Rtn4) protein family—in skeletal muscle inflammation and suggested that increased Nogo-A expression could accelerate the progression of motor innervation in an ALS animal model. Furthermore, Bruneteau *et al.* [32] revealed that Nogo-A is expressed in type I fibers and participates in the degeneration of NMJs in patients with ALS, suggesting that NMJ alterations could be prognostic indicators of motor impairment and neurodegeneration.

In this study, we demonstrated that CS treatment improved motor function and attenuated the expression of inflammatory and oxidative stress-related proteins in the muscles and spinal cords of hSOD1$^{G93A}$ mice. The observed changes in body and muscle weights were as expected, with Tg mice weighing less than age-matched nTg mice and consistent with the progression of muscular atrophy in hSOD1$^{G93A}$ mice. Notably, weight differences help distinguish between presymptomatic and symptomatic animals. We detected impaired motor function in the footprint test, while CS treatment increased body and muscle weights, although the difference was not statistically significant. The increase in GC muscle weight is partially related to improving muscle function as the GC contributes to the function of other "fast" movements and energy metabolism in hSOD1$^{G93A}$ mice. Future experiments are required to understand further the relationship between glucose metabolism and mitochondrial function in the muscle and confirm the relationship between GC muscle weight gain and motor function in CS-treated hSOD1$^{G93A}$ mice. Additionally, CS prevented the loss of motor neurons in the SP of the hSOD1$^{G93A}$ mice. Our findings suggest that CS treatment augments anti-inflammatory events, improving motor function and the survival of motor neurons in the muscles and spinal cords of hSOD1$^{G93A}$ mice. These results are consistent with those of previous studies on the biological activities of CS [17,18], including antioxidant, antitumor, anti-hepatitis, antimicrobial, immunoregulatory, anti-influenza, and anti-Parkinson effects [33–37].

ALS is a complex disease involving multiple pathogenic mechanisms and interrelated pathways, including mitochondrial dysfunction, oxidative stress, and autophagic dysfunction. Autophagy regulates skeletal muscle remodeling and maintains muscle function during movement. In addition, neurogenic atrophy is induced by

skeletal muscle denervation through the suppression of autophagy. Furthermore, muscle regeneration is involved in autophagic flux, and autophagy contributes to mitochondrial regeneration [38]. The role of dysregulation of autophagy in ALS is well understood. Mutations in the genes encoding SQSTM1/p62, TDP-43, and OPTN [39] contribute to the progression of ALS [40]. p62/SQSTM1 is associated with polyubiquitinated proteins and degraded by autophagy. Simultaneously, TDP-43 aggregation can be eliminated by autophagic flux and autophagosome formation either via mTOR inhibition-dependent or -independent pathways. Indeed, mTOR signaling pathway regulates autophagy and muscle regeneration. Although various drugs have been developed that modulate autophagy, many gaps exist in our understanding of the molecular mechanisms underlying their effects. Nevertheless, mTOR inhibitors are currently being evaluated in ALS clinical trials; however, to our knowledge, no results have been published. Our study shows that CS regulates autophagy in muscles; however, whether CS affects muscle regeneration and neuromuscular connections in hSOD1$^{G93A}$ mice remains to be determined. Moreover, future studies are needed to unravel the relationship between muscle metabolism and autophagy regulation by CS in hSOD1$^{G93A}$ mice and expand investigations to other animal models of ALS. Furthermore, additional studies investigating the effects of CS in the muscles and SP of other ALS animal models and the survival rate in CS-treated groups are warranted to consider CS treatment in patients with ALS [5].

## 5. Conclusions

In this study, we demonstrated that CS improved motor function and enhanced anti-inflammatory events both in the muscles and spinal cord of hSOD1$^{G93A}$ mice. Additionally, CS inhibited motor neuron death in the SP and regulated autophagy in the muscles of hSOD1$^{G93A}$ mice. The results suggest that CS can delay disease progression and the onset of a systemic disease, such as ALS. However, further studies are needed to investigate the bioactive compounds of CS for the development of drug candidates for ALS treatment.

## Availability of Data and Materials

All data are available within the article and available by reasonable request.

## Author Contributions

EJY conceptualized the study, performed the animal procedures, and reviewed and edited the manuscript. SHL performed biochemical and molecular studies and analyzed the data. Both authors contributed to editorial changes in the manuscript. Both authors have read and agreed to the published version of the manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

The Institutional Animal Care Committee of the Korea Institute of Oriental Medicine approved the experimental protocol (KIOM protocol # 17-061), and all experiments were performed in accordance with the U.S. National Institutes of Health guidelines and guidelines of the Animal Care and Use Committee at the KIOM.


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Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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