의학박사 학위논문

Neuroprotective Effect of JGK-263 in Transgenic SOD1-G93A Mice Model of Amyotrophic Lateral Sclerosis

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Neuroprotective Effect of JGK-263 in Transgenic SOD1-G93A Mice Model of Amyotrophic Lateral Sclerosis

By

Suk-Won Ahn

A thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Neuroscience) at Seoul National University College of Medicine

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ABSTRACT

Neuroprotective Effect of JGK-263 in Transgenic SOD1-G93A Mice Model of Amyotrophic Lateral Sclerosis

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Introduction: Glycogen synthase kinase-3β (GSK-3β) activity plays a central role and has been identified as one of the important pathogenic mechanisms in the regulation of a wide range of cellular functions, and additionally, GSK-3β inhibitor has been investigated as a modulator of apoptosis and has been shown to confer significant protective effects on cell death in various neurodegenerative diseases. Especially, GSK-3β inhibitors have been shown to prolong motor neuron survival and suppress disease progression of amyotrophic

lateral sclerosis (ALS). In this study, we evaluated the neuroprotective effects of a new GSK-3β inhibitor, JGK-263, on ALS in G93A superoxide dismutase 1 (SOD1) transgenic mice as a promising novel therapeutics.

Method: Before *in vivo* study, the biochemical efficacy of a new GSK-3β inhibitor, JGK-263, was evaluated via treating mouse motor neuron-like hybrid cells (NSC-34) with low to high doses of JGK-263 after 48 h of serum withdrawal. And biochemical changes of the apoptotic pathway components, including cleaved caspase-3, cytochrome *c*, Fas, Fas-associated protein with death domain (FADD), and cleaved caspase-8 were measured. Then JGK-263 was administered orally to 93 transgenic mice with the human G93A-mutated SOD1 gene. The study was designed into three groups: a group administered 20 mg/kg JGK-263, a group administered 50 mg/kg JGK-263, and a control group not administered with JGK-263. Clinical status, rotarod test, onset of symptom and survival of transgenic mice with ALS were evaluated. In addition, sixteen mice from each group were selected for further biochemical studies of motor neuron count, apoptosis,

and cell survival signals.

Results: An *in vitro* study on NSC-34 motor neuron revealed that treatment with JGK-263 reduced serum-deprivation-induced motor neuronal apoptosis, The cell viability was peaked after treatment of serum-deprived cells with 50 μM JGK-263. In *vivo* study in the SOD1-G93A transgenic mice, JGK-263 administration remarkably improved motor function and prolonged the time until symptom onset, and rotarod failure, and survival in transgenic SOD1-G93A mice with ALS compared to control mice. In both 20 mg/kg and 50 mg/kg JGK-263 treated groups, choline acetyltransferase (ChAT) staining in the ventral horn of the lower lumbar spinal cord showed a large number of viable motor neurons, suggesting normal morphology. The neuroprotective effects of JGK-263 in ALS mice were also identified by western blot analysis of spinal cord tissues in transgenic ALS mice. The apoptosis signals were reduced by JGK-263 administration in G93A transgenic ALS mice. By contrast, the levels of phosophoinositide 3-kinase (PI3K) p85 and phosphorylated Akt (p-Akt), both of which are cell survival markers, in the spinal cords were elevated in G93A

transgenic ALS mices treated with JGK-263 compared to control mice

Conclusions: These studies suggest that JGK-263, a novel oral GSK-3β inhibitor, has a neuroprotective effect through modulation of the apoptosis and cell survival pathway in motor neuron degeneration of the NSC-34 cells and SOD1-G93A transgenic mice. Therefore the JGK-263 administration could be a promising therapeutic strategy for ALS.

Key words: Amyotrophic lateral sclerosis, JGK-263, Glycogen synthase kinase-3β (GSK-3β), SOD1-G93A, NSC-34 cell

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LIST OF ABBREVIATIONS

AD, Alzheimer's disease

ALS, amyotrophic lateral sclerosis

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid

ANG, angiogenin

CCK-8, Cell Counting Kit-8

ChAT, Choline acetyltransferase

CREB, cAMP response element binding

Cu/Zn-SOD1, copper/zinc ion-binding superoxide dismutase 1

DMEM, Dulbecco's modified Eagle medium

FADD, Fas-Associated protein with Death Domain

FBS, fetal bovine serum

FasL, Fas ligand

FUS, fusion in sarcoma

GSK-3β, glycogen synthase kinase-3β

LMN, lower motor neuron

NMDA, N-methyl-D-aspartate

NSC-34, mouse motor neuron-like hybrid cells

OPTN, optineurin

PD, Parkinson's disease

p-Akt, phosphorylated Akt

PARP, poly ADP-ribose polymerase

PI3K, phosphatidylinositol 3-kinase

PK, pharmacokinetic

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

TARDBP, TAR DNA binding protein

Tg, transgenic

UMN, upper motor neuron

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive deterioration of motor neurons resulting in motor weakness, muscular atrophy, swallowing difficulty, and respiratory distress (1–4). The clinical hallmark of ALS is the presence of both upper motor neuron (UMN) and lower motor neuron (LMN)–related symptoms involving the brainstem and multiple spinal cord regions of innervations (1–7). UMN symptoms involving the limbs and bulbar symptoms lead to spasticity, weakness, brisk deep tendon reflexes, and spastic dysarthria. Spastic dysarthria is characterized by slow and distorted speech and pathologically brisk gag reflex and jaw jerk. By contrast, LMN–related features include fasciculation, cramping, muscular wasting, muscular weakness, flaccid dysarthria, and dysphagia. Progressive weakness of the respiratory muscles leads to respiratory failure, eventually leading to dyspnea, orthopnea, hypoventilation with hypercapnia, and early morning headaches in most cases of ALS (1–7). Therefore, research on pathomechanisms, specific diagnostic markers, and innovative therapies may be significant in extending life expectancy of

patients with ALS (2, 3, 5–8).

Although the underlying mechanisms of motor neuron degeneration and therapeutic intervention for ALS remain uncertain, the pathomechanisms underlying the development of ALS seem to be multifactorial, with emerging evidence of involvement of programmed cell death, oxidative stress injury, misfolded protein aggregation, altered axonal transport, impaired calcium metabolism, increased glutamate activity, and dysfunctional mitochondria (1–3, 8–11).

Mutations in Cu/Zn–SOD1 (copper/zinc ion–binding superoxide dismutase 1), TARDBP (TDP–43; TAR DNA binding protein), FUS (fusion in sarcoma), ANG (angiogenin), and OPTN (optineurin) lead to typical clinical phenotypes of ALS (2, 3, 8, 10–12). Although the pathophysiology remains unclear, the current understanding links genetic mutations to a toxic gain of function of the SOD1 enzyme, with generation of free radicals that eventually leads to cell injury and death. Additionally, SOD1 mutations induce conformational instability and misfolding of the SOD1 protein, resulting in the formation of intracellular aggregates that inhibit normal proteasome function, disrupting axonal transport systems and vital cellular functions

(10-12). Recently, TDP-43 and FUS, which are multifunctional proteins involved in gene expression and regulation, including the processing of small regulatory RNAs (microRNAs), RNA splicing and maturation, transport, and translation, have come into greater prominence (2, 3, 10-14). Additionally, TDP-43 was recognized as a major component of ubiquitinated cytoplasmic protein aggregates in some patients with sporadic ALS, but not in the nucleus, as seen in the normal neurons (1-3, 10-12).

Glutamate-induced excitotoxicity has been implicated in ALS pathogenesis. Glutamate is the main excitatory neurotransmitter in the CNS and binds to ionotropic N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the postsynaptic membrane (1-3, 15). Excessive activation of these postsynaptic receptors by glutamate can induce neurodegeneration by damaging intracellular organelles and upregulating proinflammatory mediators through activation of calcium dependent enzymatic pathways and generation of free radicals (1-3, 8, 15). Non-neuronal cells, such as astrocytes and microglia, might also contribute directly to

neurodegeneration by decreasing neurotrophic factors, secreting neurotoxic mediators, and modulating glutamate receptor expression (1-3, 8, 15).

Among the mechanisms proposed for motor neuron death in ALS, intracellular signaling pathways have been reported to contribute to programmed cell death or apoptosis (16-19). Glycogen synthase kinase-3β (GSK-3β) plays a significant role in intracellular apoptosis and cell survival pathways involving phosphatidylinositol 3-kinase (PI3K)/Akt signals, and hence, the dysregulation of GSK-3β has been suggested to be involved in the development of diverse neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), and ALS (16-22).

Glycogen synthase kinase-3 (GSK-3) is a ubiquitous serine/threonine kinase, which was first described as a major regulator of glycogen metabolism by phosphorylation and subsequent inhibition of glycogen synthase. It has two isoforms, GSK-3α and GSK-3β, which are highly homologous in mammals (16-18). They are inactivated by phosphorylation of Ser21 and Ser9 in GSK-3α and β, respectively, and have been implicated in various biological processes (16-22). Most

studies have focused on the β isoform due to its more established role in cell survival and viability. GSK-3β is involved in the regulation of a wide range of cellular functions including differentiation, growth, proliferation motility, cell cycle progression, embryonic development, apoptosis, and insulin response. GSK-3β promotes apoptosis by inhibiting pro-survival transcription factors, such as CREB (cAMP response element binding) and heat shock factor-1, and facilitating proapoptotic transcription factors such as p53. β-catenin, which is a critical factor in *Wnt* signaling, is phosphorylated by GSK-3β and then degraded through the ubiquitin-proteasome system. Inhibition of GSK-3β activity leads to stabilization and accumulation of β-catenin in the cytosol, which is then shuttled into the nucleus to regulate gene expression (16-25).

In ALS, the increase of GSK-3β activity has been investigated as a potential pathogenic mechanism associated with neuronal apoptosis. Previous studies in thoracic cord from sporadic ALS patients have shown significant upregulation of GSK-3β expression (26-27). Moreover, motor neurons transfected with G93A mutant human Cu/Zn-superoxide

dismutase (*h*SOD1) gene also increases GSK−3β activity (28). In addition, a recent series of data suggested that GSK−3β activity could also be an important factor in the mitochondria mediated apoptotic pathways (17, 29, 30). Therefore, many pharmaceutical and academic researchers have focused on the development of a GSK−3β inhibitor as a potential therapeutic strategy in the treatment of ALS.

A classic candidate for a nonspecific GSK−3β inhibitor is lithium (18). Lithium was found to inhibit GSK−3β in a competitive manner by binding directly to magnesium−binding sites of the enzyme, thus providing evidence for a molecular mechanism for enzyme inactivation of GSK−3β. However, development of therapeutics using existing GSK−3β inhibitors is not yet possible. In addition, most specific GSK−3β inhibitors can only be administered intraperitoneally which becomes a limitation for *in vivo* use and in human studies. Therefore, a novel GSK−3β inhibitor such as JGK−263, which can be orally administered, would be a potential therapeutic strategy for ALS. JGK−263 is a 8−amino−[1,2,4]triazolo[4,3−a]pyridin−3(2H)−one derivative that functions as a small molecular GSK−3β inhibitor by binding to the polar residues

(Lys85, Glu97, Asp200, Gln185 and Arg141) of GSK-3β, located in its binding cavity, which play an important role in binding other kinases. Previous pharmacokinetic (PK) studies revealed the effect of JGK-263 on GSK-3β enzyme activity, which was confirmed by using GSK-3β and phospho-GSK-3β antibodies (19).

In this study, we investigated the therapeutic effects and biochemical influences of JGK-263 on the apoptotic pathway of motor neurons by using a cell culture model, and eventually revealed a neuroprotective effect of JGK-263 in *h*SOD1-G93A transgenic ALS mice.

MATERIALS AND METHODS

1. Cell culture and JGK-263 treatment

Mouse motor neuron-like hybrid cells (NSC-34) were maintained in culture dishes containing Dulbecco's modified Eagle medium (DMEM) (JBI, Korea) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). Cells were kept in a humidified incubator at 37℃ under 5% CO_2 (30). Cells were subjected to serum deprivation for 48 h with and without treatment with various concentrations of JGK-263 (0, 0.2, 1, 10, 50, and 100 μM). Serum deprivation was used to induce apoptosis in NSC-34 cells.

JGK-263, a new GSK-3β inhibitor, was supplied by Jeil Pharmaceutical Drug Discovery Laboratories (R&D Center; Jeil Pharmaceutical Co., Ltd.). It showed good potency in enzyme- and cell-based assays (IC_{50} = 111 nm, EC_{50} = 1.78 μM). Moreover, it demonstrated desirable water solubility, PK profile, and moderate brain penetration (19).

2. Cell viability assay

NSC-34 cells were subcultured in 96-well plates at a density of 1.5 × 10^4 cells per well, and on the next day, cells were subjected to serum deprivation for 48 h with and without treatment with various concentrations of JGK-263. After this treatment period, cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Tokyo, Japan).

3. Western blotting and immunoreactivity

Cell lysates were prepared from all the experimental conditions, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and blotted with anti-tau (Invitrogen, Camarillo, CA, USA), anti-phospho-tau (ser 396) (Invitrogen, Camarillo, CA, USA), anti-Fas (Santa Cruz Biotech, Delaware, CA, USA), anti-Fas ligand (FasL, Santa Cruz Biotech, Delaware, CA, USA), anti-cleaved caspase-8 (Novus Biologicals, Littleton, CO, USA), anti-cleaved caspase-3 (Cell signaling, Beverly, MA, USA), and anti-cytochrome c (Cell signaling, Beverly, MA, USA) antibodies. For evaluation of cytosolic cytochrome c levels, cell suspensions were prepared according to the manufacturer's protocol (Mitochondria isolation kit for cultured cells; Thermo scientific, Rockford, IL,

USA). Protein loading was controlled by probing with β-actin (Cell signaling, Beverly, MA, USA) antibody on the same membrane. For immunoprecipitation, protein samples were incubated overnight at 4℃ with anti-Fas antibody. The complexes formed were immunoprecipitated using protein A-Sepharose. The Sepharose beads were boiled in sample buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Western blot analysis was performed using anti-Fas-Associated protein with Death Domain (FADD).

4. *h*SOD1-G93A transgenic mice

Ninety-three transgenic mice with the human G93A-mutated SOD1 gene (B6SJL-Tg (SOD1-G93A) 1 Gur/J; Jackson Laboratory, Bar Harbor, Me, USA) and sixteen wild-type mice (B6SJLF1/J; Jackson Laboratory; wild-type group) were used in this study. The transgenic mice were divided into three groups: a group administered with 20 mg/kg JGK-263 (n = 31), a group administered 50 mg/kg JGK-263 (n = 31), and a control group (n = 31). Clinical status, rotarod time, and survival of transgenic ALS mice were evaluated daily. Sixteen mice from each group were randomized for biochemical studies

and further evaluated for motor neuron survival, PI3K/Akt pathway, and apoptotic signals. The expression of unfolded protein response and apoptotic changes in *h*SOD1-G93A transgenic mice increase at 50 days as described by the Jackson Laboratory. Hence, in transgenic mice treated with JGK-263, regular doses of 20 mg/kg or 50 mg/kg were administered orally twice per day for five days per week, starting at 60 days post-birth. Non-treated transgenic mice from the control group and 16 wild-type mice were used for comparative studies. Transgenic mice used for biochemical analyses were sacrificed at 90 and 110 days after birth. All other mice were euthanized at the disease endpoint described below.

5. Clinical evaluation of disease progression in SOD1-G93A transgenic mice

Behavioral experiments were conducted in a single-blind manner. Clinical status of all transgenic mice was evaluated three times per week beginning 60 days after birth. Symptom onset was defined as the shaking of limbs when the mouse was suspended in the air by its tail, suggested to be due to the

clinical involvement of the UMN system (12, 17, 28). Clonus, hyperreflexia, and crossed spread of spinal reflexes were also detected in most mice at disease onset. Disease endpoint was defined to be when the mice were unable to stand upright within 30 s after being placed on their sides on a flat surface. At this point, the mice were killed (32). Mice were subjected to a week-long learning period at 53 days after birth, during which they performed an accelerating rotarod test. The rotarod test was performed 3 days per week. The time spent walking on the rotarod was measured, and time was averaged over 3 days. If the mean time was less than 10 s, it was defined as rotarod failure.

6. Motor neuron analysis and western blotting with using spinal cords of SOD1-G93A transgenic mice

Choline acetyltransferase (ChAT), which is a well-known marker for cholinergic motor neurons, was used to identify motor neuron cells. Transgenic animals were sacrificed at different time-points (90 days and 110 days) depending on their clinical features. Mice from each group were euthanized with pentobarbital sodium and perfused transcardially with cold

PBS followed by cold paraformaldehyde. Lumbar spinal cords were dissected, fixed in paraformaldehyde, and cryoprotected in a cold sucrose solution. Dissection was performed carefully, preserving the anatomical integrity of the spinal cord tissue, and 12-μm-thick transverse sections were obtained. Motor neurons in the ventral horn of the spinal cord were stained for choline acetyltransferase (anti-ChAT; Millipore, Billerica, MA, USA) and counted, as previously described (17). Motor neuron cells stained in the anterior horn were counted manually as their diameter was more than 10 μm. To avoid counting non-neuronal cells including glial cells, only cells with a clearly defined morphology were counted and those with perikaryal vacuoles were excluded.

To evaluate the possible mechanisms of JGK-263 action in the transgenic ALS mice model, western blotting of phospho-PI3K (p-PI3K) p85, phospho-Akt (p-Akt), phospho-GSK-3β (p-GSK-3β), and antibodies of apoptosis signaling factors were performed. For western blotting, mice were anesthetized with pentobarbital sodium and intracardiac perfusion of PBS was performed. Shortly after, the spinal cord of each mouse was quickly removed, cooled in ice-cold artificial cerebral

spinal fluid (CSF) for 5 min, and stored at −80℃. Each spinal cord was homogenized with a tissue homogenizer in 5 volumes of lysis buffer (150 mM Sodium chloride, 1% Triton X−100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris−HCl, pH 7.5, and 2 mM EDTA, protease and phosphatase inhibitors tablet). Tissue lysates were centrifuged at 13,000 rpm for 20 min, and supernatants were used to measure p−GSK−3β (Cell Signaling Tech, Beverly, MA, USA), caspase−3 (Cell Signaling Tech, Beverly, MA, USA), cytochrome *c* (Cell Signaling Tech, Beverly, MA, USA), poly ADP−ribose polymerase (PARP)−1 (Santa Cruz Biotech, Delaware, CA, USA), PI3K p85 (Santa Cruz Biotech, Delaware, CA, USA), and p−Akt (Santa Cruz Biotech, Delaware, CA, USA) levels. Protein loading was controlled by probing with β−actin (Cell signaling, Beverly, MA, USA) antibody on the same membrane.

7. Statistical analysis

The data are presented as the mean ± standard errors (SE) of more than three independent tests. Data were analyzed by Prism software (GraphPad Software, San Diego, CA, USA) using either Student's *t*−test or Tukey's multiple comparison

tests after one-way ANOVA (GraphPad Prism Software). Differences were considered statistically significant when P values were <0.05. Motor activity and survival following JGK-263 treatment in transgenic mice with ALS were analyzed using SPSS v. 19.0. Symptom onset data, rotarod times, disease endpoints, and motor neuron counts are also expressed as mean ± SE. Clinical data were analyzed using one-way ANOVAs and t-tests, and P values <0.05 were considered to be statistically significant. Probabilities of symptom onset, rotarod failure, and disease endpoint were analyzed using Kaplan-Meier curves.

RESULTS

1. Results from NSC- 34 cells

In vitro motor neuron apoptosis, induced by serum deprivation, revealed that both the intrinsic and the extrinsic apoptotic pathways were activated (33). For activating apoptosis signals, we selected 48 h serum deprivation as the treatment condition in the current studies. A CCK-8 assay was done after treatment of serum-deprived NSC-34 cells with JGK-263 (0, 0.2, 1, 10, 50, 100 μM). The mean cell viability after treatment with each concentration of JGK-263 was determined. Treatment with increasing concentrations of JGK-263 up to 50 μM increased cell viability, compared to the control (45.92 ± 5.37% survival). Treatment with 50 μM JGK-263 resulted in the highest cell viability (85.23 ± 9.91%, $P < 0.001$). The protective effect peaked with 50 μM JGK-263, but at 100 μM (32.83 ± 5.19%) these protective effects were reduced ($P < 0.001$; Figure 1). GSK-3β activity was indirectly measured by the immunoreactivity (IR) ratio of phosphorylated tau (ser396) to total tau after JGK-263 treatment. As the concentration of JGK-263 increased, the IR

ratio of phosphorylated tau (ser396) to total tau decreased as shown in Figure 2. We confirmed that JGK-263 effectively inhibited GSK-3β in NCS-34 cells, and this inhibitory action showed a dose-dependent pattern ($P < 0.001$; Figure 2).

Next, we investigated late stage apoptosis by monitoring levels of cleaved caspase-3. A significant reduction in the cleaved caspase-3 signal in a dose-dependent manner was noted in groups treated with concentrations of JGK-263 above 10 μM, compared with the control group ($P < 0.001$; Figure 3). As shown in Figure 4, treatment of NSC-34 cells with JGK-263 also significantly decreased levels of cytochrome *c* in the cytosolic fraction in a dose-dependent manner ($P < 0.001$; Figure 4).

To evaluate the effect of serum deprivation in NSC-34 cells and the effect of JGK-263 treatment on the external apoptotic pathway, levels of Fas, FADD, and cleaved caspase-8 were investigated by Western blot analysis. In cells treated with JGK-263, cleaved caspase-8 decreased in a dose-dependent manner. Cleaved caspase-8 was significantly decreased after treatment with 50 μM (0.7 ± 0.06, $P < 0.01$) and 100 μM (0.67 ± 0.05, $P < 0.01$) JGK-263 (Figure 5).

However, we determined that JGK-263 treatment did not induce significant changes in the levels of Fas/FasL activation, which constitutes the first component of the extrinsic apoptosis pathway, in serum-deprived cells (Figure 6A and B). Furthermore, to identify changes in the extrinsic apoptosis pathway represented by Fas-FADD interaction, we performed immunoprecipitation. We immunoprecipitated the NSC-34 cell lysates with Fas antibody, followed by western blotting using a FADD antibody as the probe. We confirmed that Fas-FADD interactions were significantly decreased in cells treated with JGK-263 ($P < 0.001$; Figure 6C). This finding demonstrates that the effect of JGK-263 on extrinsic pathway is relevant to intracellular region adjacent to the FAS-FADD interaction.

2. Results from *h*SOD1-G93A transgenic mice

Clinical evaluation involving rotarod test revealed that motor performance and strength in hSOD1-G93A transgenic mice were increasingly impaired over time. However, the level of motor function deficit significantly differed among transgenic mice groups (Table 1). Statistical analysis showed a remarkable improvement in motor function deterioration after

JGK-263 administration in transgenic mice with ALS. Symptom onset (Table1 and Figure 7, $P < 0.01$) and rotarod failure (Table 1 and Figure 8, $P < 0.05$) were significantly delayed, and survival time (Table1 and Figure 9, $P < 0.05$) was longer in the JGK-263 treated groups than in the control group. Furthermore, administering JGK-263 at a higher dose of 50 mg/kg led to the most prominent effects. These findings indicate that JGK-263 administration exerts a therapeutic effect on transgenic ALS mice with the G93A-mutant hSOD1 gene by delaying disease progression.

To evaluate motor neuron function in the lumbar spinal cord, ChAT staining was performed at days 90 and 110, because hSOD1-G93A transgenic mice usually show prominent abnormal spinal cord pathology of neurofilament-rich spheroids and significant motor neuron loss beyond 3 months of age; which were described on the Jackson Laboratory. In the JGK-263 groups, more ChAT-positive motor neurons were found in the ventral horn of the lower lumbar spinal cord showing normal morphology (Figure 10). Additionally, measurement of motor neuron count at different stages of disease progression showed more motor neurons in the JGK-263 groups at both 90 and 110

days (Table 2 and Figure 11, $P < 0.01$).

Western blot analysis showed decreased levels of cytochrome *c* (cytosol form), cleaved caspase-3, and cleaved PARP-1 in the spinal cords of 102-day-old hSOD1-G93A transgenic ALS mice treated with JGK-263 as compared to control mice (Figure 12). These findings indicate that apoptosis signals can be inhibited by JGK-263 administration in hSOD1-G93A transgenic ALS mice. By contrast, the levels of PI3K p85 and p-Akt, both of which are cell survival markers, in the spinal cords were elevated in 102-day-old hSOD1-G93A transgenic ALS mice treated with JGK-263 as compared to control mice (Figure 13). Expression levels of proteins were also verified by mean values of window area ratio (cytosol cytochrome *c* - 3.1344 vs. 1.4101; cleaved caspase-3 - 24.989 vs. 14.745; PI3K p85 - 0.8475 vs. 1.4204; p-Akt - 0.5905 vs. 0.7171, between control and treatment group, respectively.)

DISCUSSION

In the present study, we found that JGK-263, a new GSK-3β inhibitor, delayed motor function deterioration and extended survival in *h*SOD1-G93A transgenic mice. Also, our finding that motor neuronal cell death induced by the G93A mutation was relieved by JGK-263 treatment suggests that JGK-263 has therapeutic effects on cell death associated with ALS. Possible pathomechanisms related to the therapeutic effects of JGK-263 treatment were activation of the PI3K/Akt pathway and inhibition of the apoptosis pathway, which was verified by the decrease of cytochrome *c*, cleaved caspase-3, and PARP-1.

JGK-263 more effectively inhibits GSK-3β activity in enzyme and cell-based assays than do other soluble GSK-3β inhibitors, and has optimal water solubility, bioavailability, plasma concentration, liver microsomal stability, clearance, and brain penetration (19).

In our study, we used NSC-34 motor neuron cells to evaluate the protective effect of JGK-263 and determined that treatment with JGK-263 reduced serum deprivation induced cell death via inactivation of the apoptosis pathway. These *in*

vitro results suggest that JGK-263 has a neuroprotective effect against motor neuron degeneration through modulation of the apoptosis pathway.

Usually, apoptosis can occur in two different ways: one results from the activation of the intrinsic apoptosis pathway, which brings about mitochondrial disruption, and the other, by initiating the death receptor-mediated extrinsic apoptosis pathway. In most previous reports, investigations of GSK-3β inhibitor focused on its protective effect via the modulation of intrinsic apoptosis pathways (40-43). And, this anti-apoptotic potential of GSK-3β inhibition has been known to be effective and a possible therapeutic approach to treat many neurodegenerative diseases including ALS (40-43).

By contrast, interestingly, we showed that JGK-263 is a unique compound that acts effectively on both the apoptosis pathways. In the biochemical analysis in NSC-34 cells, we tested for several apoptosis markers including casepase-3, cytochrome *c*, Fas, FasL, caspase-8, and FADD. In the absence of serum, we found that treatment with different doses of JGK-263 decreased the amount of cleaved caspase-3 and cytochrome *c,* which are the central mediators of the

mitochondrial/intrinsic apoptotic pathway (43, 44). Additionally, treatment with JGK-263 significantly decreased levels of caspase-8 in the NSC-34 cells, which is involved in the extrinsic apoptosis pathway. Collectively, these *in vitro* observations suggest a significant effect of JGK-263 in both intrinsic and extrinsic apoptosis pathways in motor neuron cells.

However, treatment with different doses of JGK-263 did not influence Fas and FasL while the amount of cleaved caspase-8 was decreased in a dose dependent manner. In addition, the relative IR ratio of Fas-FADD interaction, as determined by western blotting and immunoprecipitation, was decreased after treatment with specific doses of JGK-263. These changes of extrinsic markers corresponding to JGK-263 concentrations may provide an explanation for the results from the viability assay. Administration of JGK-263 above 10 μM inhibited the Fas-FADD interaction, although the inhibitory effect of JGK-263 was lost at 100 μM. Unfortunately, we could not demonstrate the association between JGK-263 administration and extrinsic apoptotic pathway in detail, and this is a limitation of this study, which needs to be addressed in the future. Nevertheless, the data from our *in vitro* study suggest

that JGK-263 may be effective in preventing motor neuron cell death.

Further evaluation of the therapeutic effects of JGK-263 in ALS mice with the *h*SOD1-G93A gene showed that repeated oral administration of JGK-263 in ALS mice delayed the symptom progression and extended the survival in treated groups relative to the control group. Furthermore, we showed that JGK-263 administration alleviated motor neuron deterioration in the spinal cord and affected intracellular signals involving PI3K, p-Akt, cytochrome *c*, cleaved caspase-3, and PARP-1.

The PI3K /Akt pathway is involved in controlling various critical cell responses and several studies have established that the PI3K/Akt pathway promotes cell survival, especially in neuronal cells, by enhancing the expression of anti-apoptotic proteins and inhibiting the activity of pro-apoptotic ones (41-47). In addition, PI3K activates the downstream target Akt /protein kinase B that mediates several biological effects. Some reports have shown that mutant SOD1 protein can suppress PI3K/Akt pathway activity and *h*SOD1 (G93A)-transfected motor neuronal cells are also more vulnerable to death due to

PI3K/Akt inhibition (41−48). Our results showed an increased expression of PI3K/Akt after JGK−263 administration, therefore, suggesting that the therapeutic effects of JGK−263 may be due to its direct effect on the PI3K/Akt pathway.

Additionally, for the biochemical analysis on apoptosis pathway, we tested for the apoptosis markers including casepase−3, cytochrome *c*, and PARP cleavage with using spinal cords of transgenic *h*SOD1−G93A mice. Along with the results from NSC−34 motor cells, we identified that JGK−263 administration led to the inhibition of the apoptosis pathway in the spinal cords by reducing cytochrome *c* release, caspase−3 activation, and PARP cleavage. This result indicates that JGK−263 plays a therapeutic role via the downregulation of intracellular apoptosis, which is consistent with previous findings (48).

In this study, although we did not evaluate other mechanisms of ALS involving the microglial activation, inflammation, axonal transport, autophagy pathway, protein aggregation, glutamate excitotoxicity, and neurotrophic factors, our results show that JGK−263 plays a therapeutic role by inhibiting the activity of GSK−3β in ALS pathogenesis.

Furthermore, the therapeutic effect of JGK-263 could be mediated by the activation of the PI3K/Akt pathway and the inhibition of apoptotic pathway. Therefore, the present study suggests that JGK-263 administration could be a promising neuroprotective strategy for treating ALS.

TABLES

Table 1. Clinical analysis of SOD1−G93A transgenic mice

Summary of symptom onset, rotarod failure and disease endpoint in *h*SOD1 G93A transgenic mice of ALS. JGK−263 administration showed a remarkable improvement in motor function deterioration in transgenic mice with ALS. Symptom onset and rotarod failure were significantly delayed, and survival time was longer in the JGK−263 groups than in the control group.

	Tg mice (control)	Tg mice with 20mg/kg JGK	Tg mice with 50mg/kg JGK	*P*
Symptom onset(days)	100.1±2.4	106.9±1.6	110.5±1.7	0.002
Rotarod failure(days)	122.8±1.9	129.1±2.7	130.7±2.3	0.045
Disease endpoint(days)	130.1±1.7	137.1±2.6	138.8±2.5	0.026

Data are expressed as mean values (± standard error)

The results were analyzed by ANOVA, significance: $P < 0.05$

Tg mice; SOD1−G93A transgenic mice

Table 2. Motor neuron analysis in spinal cords of SOD1-G93A transgenic mice

Quantitative data expressing motor neurons shows significant difference among the group. In the JGK-263 groups, more motor neurons were found in the ventral horn of the lower lumbar spinal cord relative to control group.

Days	Tg mice (control)	Tg mice with 20mg/kg JGK	Tg mice with 50mg/kg JGK	*P*
		*Motor neuron counts**		
90days	14.2±0.8	16.9±0.5	22.4±0.5	< 0.01
110days	9.7±0.4	14.9±0.4	16.1±0.6	< 0.01

Data are expressed as mean values (± standard error)

The results were analyzed by ANOVA, significance: $P < 0.01$

*: motor neurons were evaluated by the choline acetyltransferase (ChAT) staining in the lumbar spinal cords.

Tg mice; SOD1-G93A transgenic mice

FIGURES

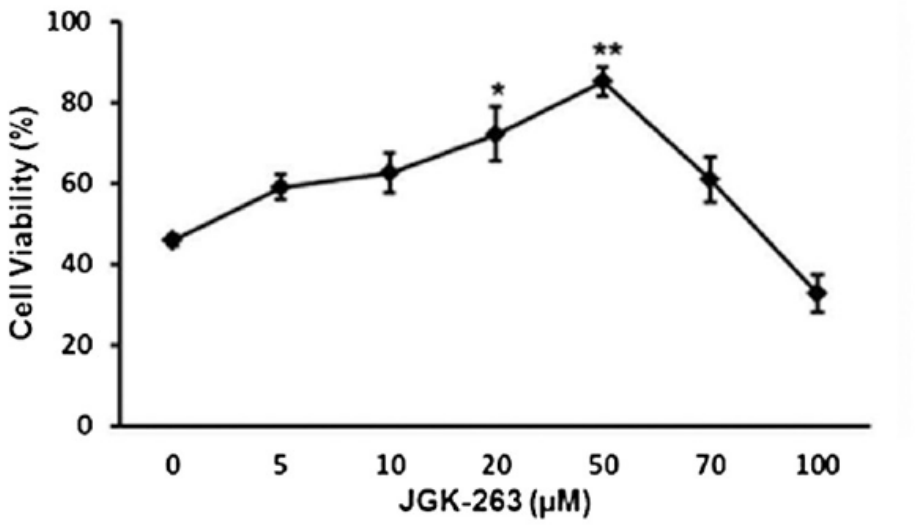

Figure 1. CCK-8 assay after JGK-263 treatment of 48 h serum-deprived NSC-34 cells

Cell viabilities after treatment of cells with each JGK-263 concentration were determined and are shown as mean (% of the cell viability in normal condition) ± standard error. The protective effect peaked after treatment with 50 μM JGK-263; however, above 100 μM, these protective effects were reduced. (*$P < 0.01$, **$P < 0.001$)

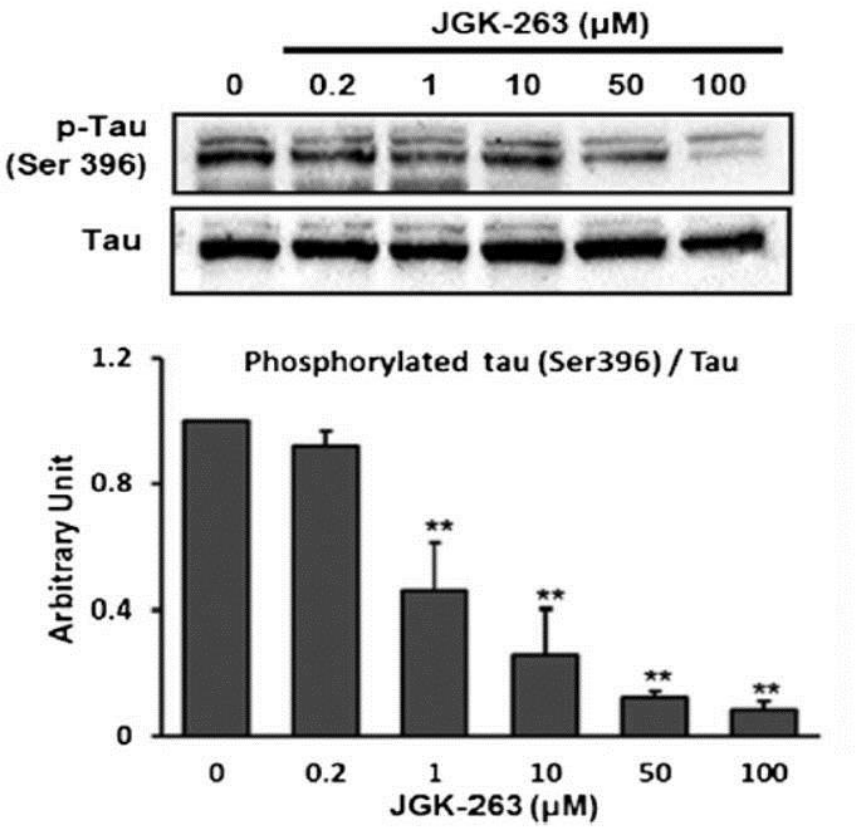

Figure 2. Immunoreactivity ratio of phosphorylated tau to total tau after JGK-263 treatment

NSC-34 cells were incubated in serum-deprived media with JGK-263. Western blotting for the phosphorylated tau and total tau with each concentration of JGK-263 is indicated. As the dose of JGK-263 increased, the immunoreactivity ratio of phosphorylated tau to total tau decreased. Quantitative data of the immunoreactivity ratio is presented as arbitrary units. (*$P <$ 0.01, **$P < 0.001$)

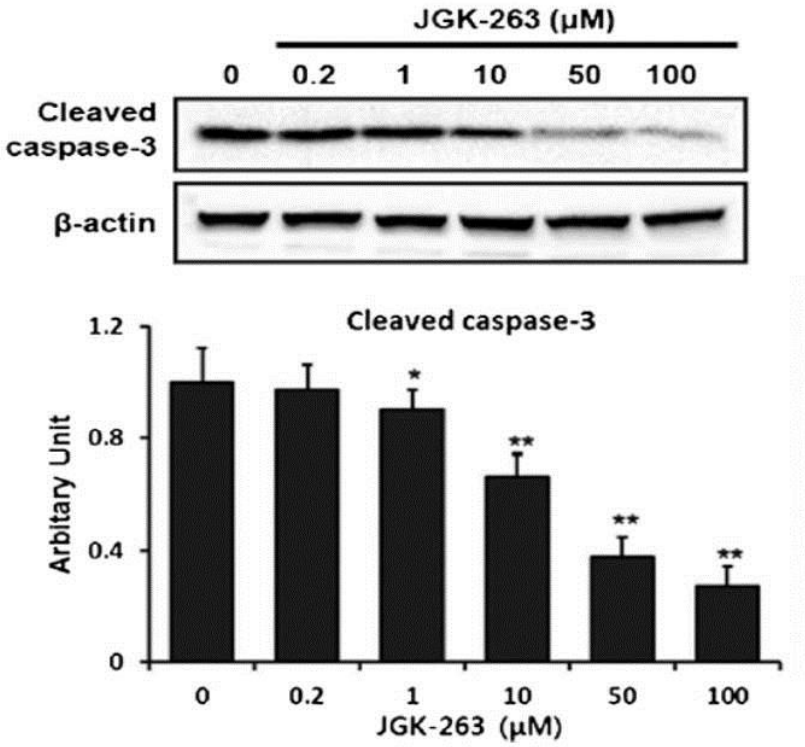

Figure 3. Cleaved caspase-3 assessed by western blotting in the NSC-34 cells after JGK-263 treatment

Quantitative data of cleaved caspase-3 immunoreactivity is expressed in arbitrary units, normalized to the loading control. Significant reductions in cleaved caspase-3 signals were noted. Data shown represent the mean ± standard error and compared with cells treated with serum-deprivation only. β-actin was used as the loading control. ($*P < 0.01$, $**P < 0.001$)

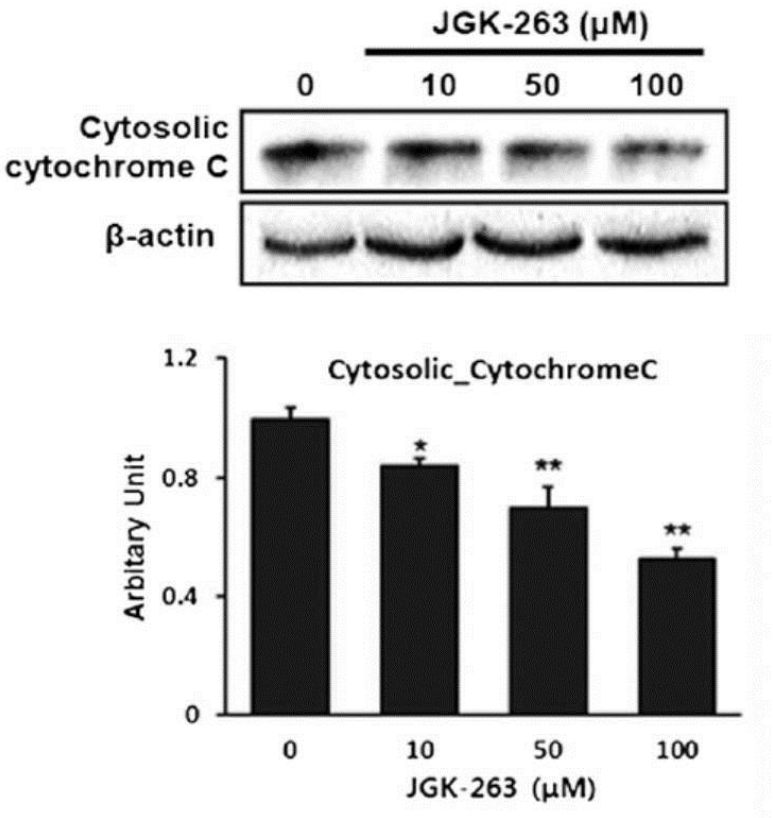

Figure 4. Cytochrome *c* assessed by western blotting in the NSC-34 cells after JGK-263 treatment

Significant reductions of cytosolic cytochrome *c* signals were noted to occur in a dose-dependent manner. Data shown represent the mean ± standard error and compared with cells treated with serum-deprivation only. (*$P < 0.05$, **$P < 0.001$)

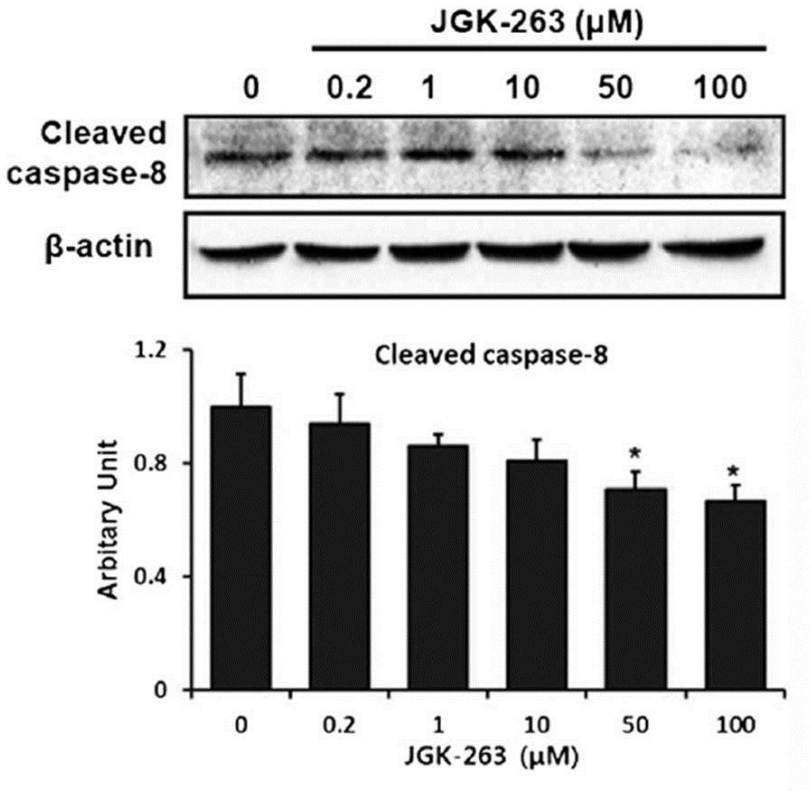

Figure 5. Caspase-8 assessed by western blotting in the NSC-34 cells after JGK-263 treatment

Immunoreactivity of cleaved caspase-8 decreased in dose-dependent manner. Data shown represent the mean ± standard error and compared with cells treated with serum-deprivation only. ($*P < 0.01$)

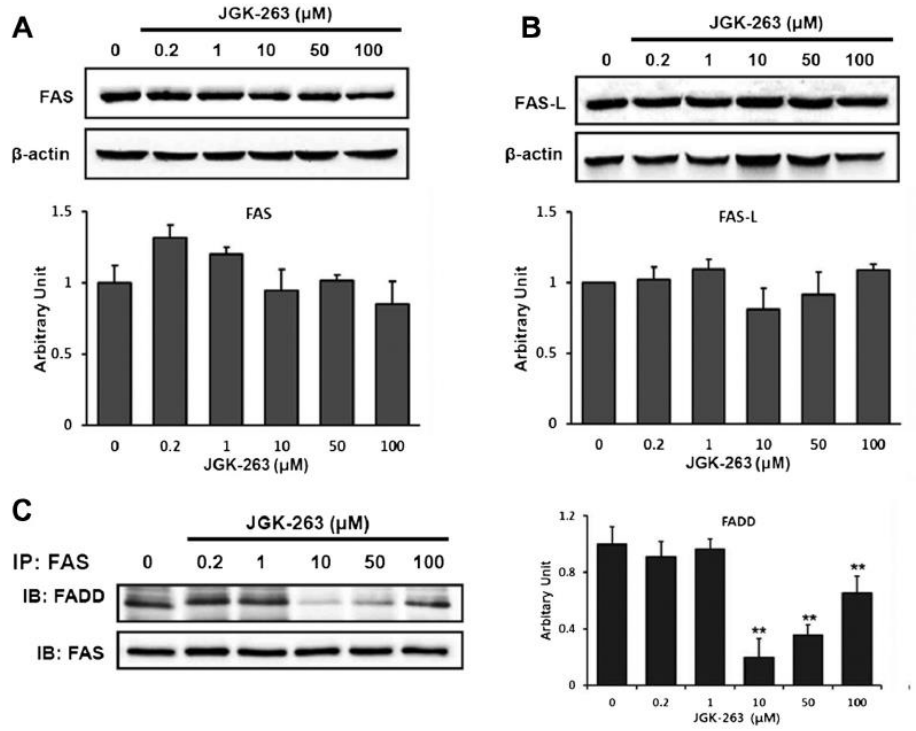

Figure 6. Extrinsic apoptosis assessed by western blotting in the NSC-34 cells after JGK-263 treatment

Fas, Fas ligand did not show any significant changes in immunoreactivity in response to different JGK-263 concentrations (A, B). By means of immunoprecipitation of NSC-34 cell lysates with Fas antibody, followed by Western blotting and probing with an anti-FADD antibody, interaction of Fas-FADD were found to be significantly decreased in the JGK-263 treated cells (C). ($*P < 0.05$, $**P < 0.001$)

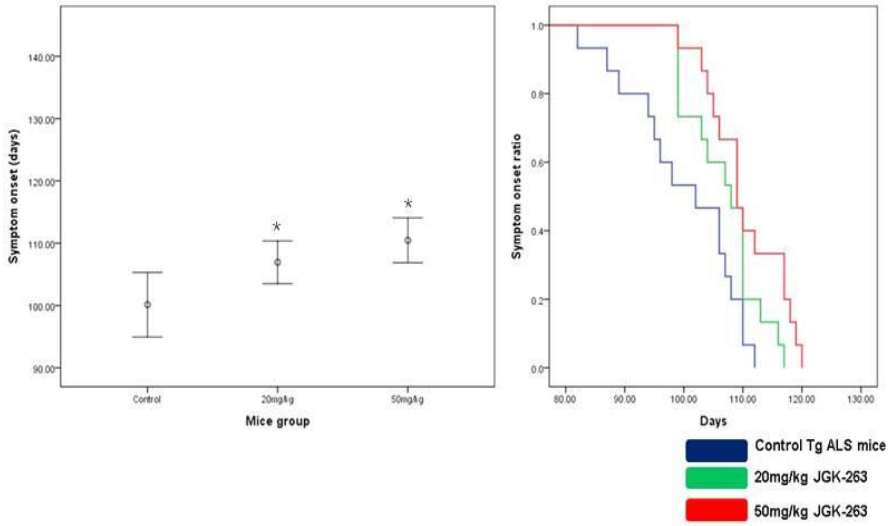

Figure 7. Onset of clinical symptoms in SOD1-G93A transgenic mice

Clinical evaluation involving rotarod test revealed that motor performance and strength in G93A transgenic mice were increasingly impaired over time. However, the level of motor function deficit significantly differed among SOD1-G93A mice groups. By the JGK-263 administration, symptom onset of transgenic mice with ALS was significantly delayed compared with the control group. The results were analyzed by ANOVA. ($*P < 0.05$)

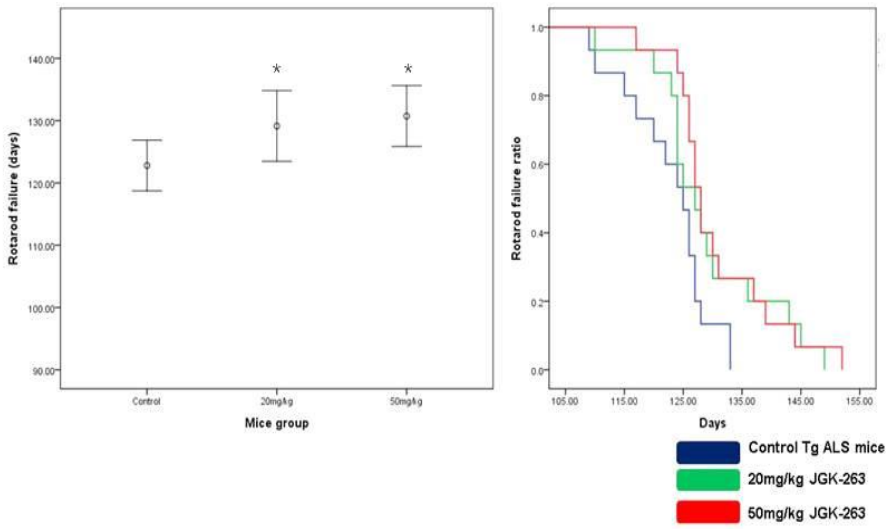

Figure 8. Time of rotarod failure in SOD1-G93A transgenic mice

By the JGK-263 administration, time of rotarod failure was significantly delayed compared with the control group. The results were analyzed by ANOVA. ($*P < 0.05$)

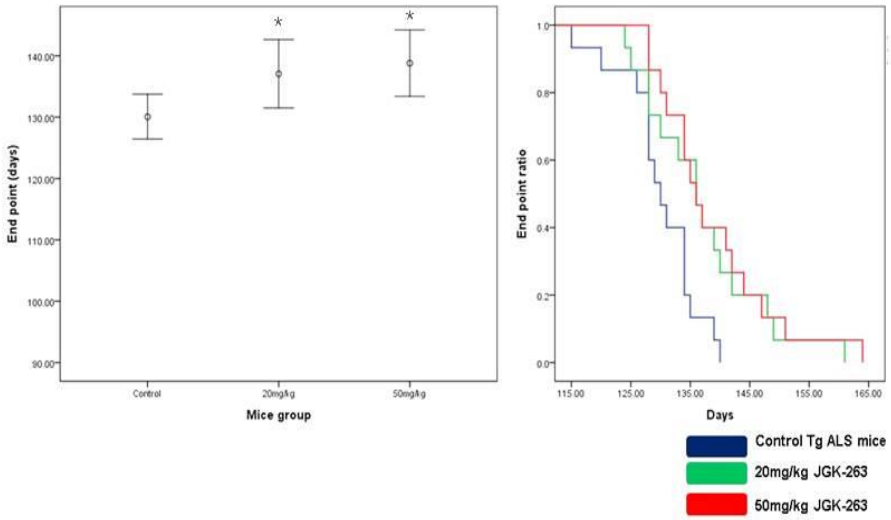

Figure 9. Time of disease endpoint in SOD1-G93A transgenic mice

By the JGK-263 administration, time of disease endpoint (survival) was significantly delayed compared with the control group. The results were analyzed by ANOVA. ($*P < 0.05$)

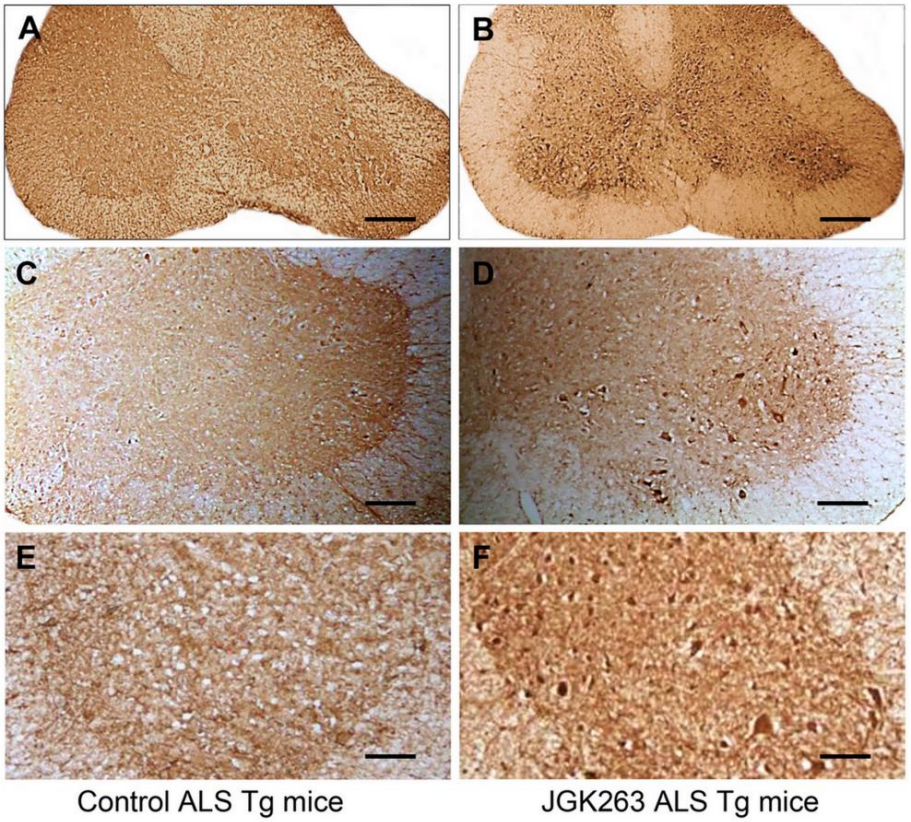

Figure 10. Motor neurons in the ventral horn of spinal cord of SOD1-G93A transgenic mice

This slides revealed motor neurons in transgenic ALS mice by choline acetyltransferase (ChAT) staining in the ventral horn of the lower lumbar spinal cord. In the JGK-263 groups (B, D, F), more much motor neurons were found in the ventral horn of the lower lumbar spinal cord, and they showed normal morphology compared with control mice (A, C, E). Scale bars: A&B, 100 μm; C&D, 60 μm; E&F, 40 μm.

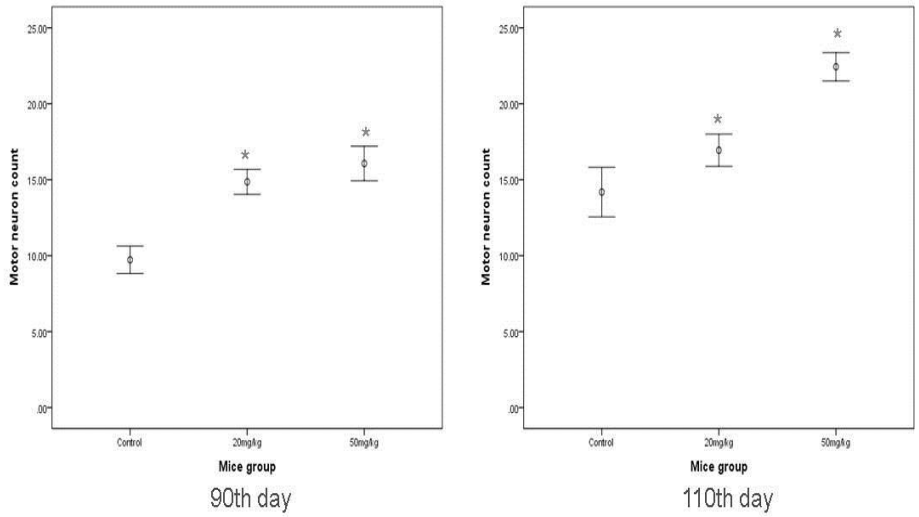

Figure 11. Quantitative data expressing counts of motor neurons in SOD1-G93A transgenic mice

The measurement of motor neuron count at different stages of disease progression showed more much motor neurons in the JGK-263 groups at both day 90 and day 110. The results were analyzed by ANOVA. ($*P < 0.05$)

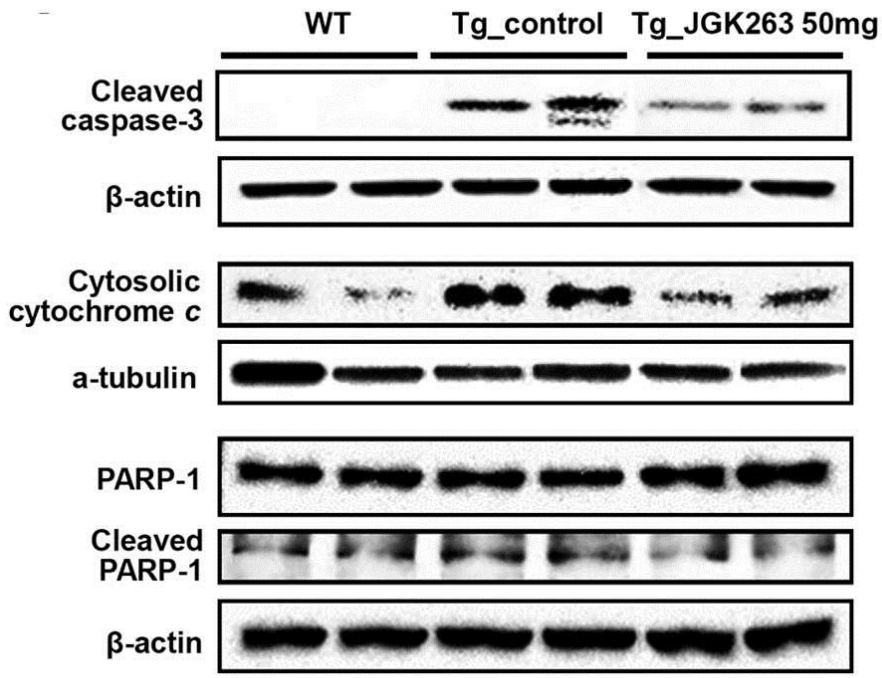

Figure 12. Apoptotic signals assessed by western blotting in SOD1-G93A transgenic mice

Western blot analysis showed decreased levels of cleaved caspase-3, cytochrome *c* (cytosol form), and cleaved PARP-1 in the spinal cords of 110-day-old SOD1-G93A transgenic ALS mice treated with JGK-263 compared to control mice. These findings indicate that apoptosis signals can be inhibited by JGK-263 administration in SOD1-G93A transgenic ALS mice.

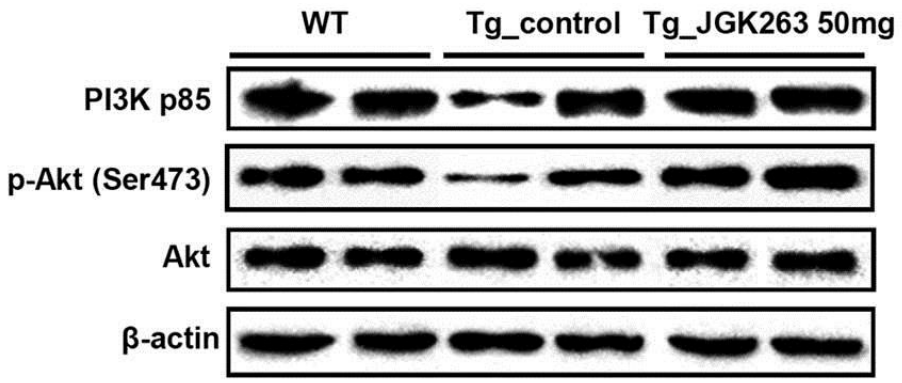

Figure 13. PI3K/Akt pathway assessed by western blotting in SOD1-G93A transgenic mice

Western blot analysis showed increased levels of PI3K and p-Akt, both of which are cell survival markers, in the spinal cords were elevated in 110-day-old SOD1-G93A transgenic ALS mice treated with JGK-263 compared to control mice.

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국문 초록

서론: Glycogen synthase kinase-3β (GSK-3β) 는 여러 가지 질환들에서 세포생존 및 사멸과 관련된 다양한 기능을 맡고 있고, 그 중에서도 신경세포의 손상과 퇴행 과정에서 중요한 병태생리학적 역할을 한다는 것이 알려져 있다. 최근에는 GSK-3β 활성 억제제가 근위축성측삭경화증을 포함한 신경 퇴행성 질환들에서 신경세포 손상을 최소화하고 질병의 진행을 억제하는 효과가 보고되면서, 향후 신경계 질환들의 치료제 가능성에 대해 많은 연구가 진행되고 있다. 이번 연구에서는 새로운 GSK-3β 활성 억제제인 JGK-263를 SOD1-G93A 형질전환 마우스에 경구 투여함으로써 운동신경세포의 보호 효과와 궁극적으로는 질병의 진행을 억제할 수 있는 치료 후보 물질로서의 가능성을 연구하고자 한다.

방법: SOD1- G93A 형질전환 마우스를 이용하여 실험을 진행하기 전에 NSC-34 운동신경세포주에서 JGK-263을 처치하여 세포 생존능의 변화, 세포사멸 과정에 미치는 영향 등에 대해 연구를 진행하였다. 특히 세포사멸과 관련하여 cleaved caspase-3, cytochrome *c*, Fas, FADD, cleaved caspase-8 등과 같은 신호전달 단백질들을 웨스턴 블롯을 통해 분석하였다. 생체내 실험을 위

해 SOD1- G93A 형질전환 마우스에 JGK-263을 경구로 투여하였고, 총 93마리의 형질 전환 마우스를 사용하여 대조군, 20 mg/kg JGK-263 투여군, 50 mg/kg JGK-263 투여군 등의 3개 군으로 나누었다. 생후 60일째에 JGK-263 약물이 처음 투여 되었고, 그 후 정기적으로 형질전환 마우스들의 운동기능을 검사하여 증상의 발현 시기 및 로타로드 실패 시기, 생존기간 등을 확인하였다. 또한 90일째와 110일째에는 각 군당 16마리의 마우스들을 선정하여 척수 조직에서 운동신경세포의 숫자 및 형태를 면역조직염색을 통해 확인하는 한편, 세포내 신호 전달 과정 및 세포사멸 등에 대한 생화학적 실험들을 시행하였다.

결과: NSC-34 운동신경세포주의 실험 결과 JGK-263 은 운동신경세포의 생존능을 향상시키는 효과를 보였고, 특히 50 μM 농도에서 가장 우수한 보호 효과를 보임을 관찰할 수 있었다. 그리고, JGK-263 는 농도의존적으로 cleaved caspase-3, cleaved caspase-8 그리고 cytochrome *c* 등의 유리를 감소시킴을 관찰할 수 있었다. 특히 JGK-263 투여로 Fas-FADD 상호작용능력이 감소되는 것을 관찰할 수 있었는데, 이는 cleaved caspase-8 의 활성도 억제의 원인 기전으로 분석된다.

SOD1-G93A 형질전환 마우스를 이용한 동물 실험에서는, JGK-263 투여가 SOD1-G93A 형질전환 마우스들의 운동 기능이 현격

히 저하되는 시기를 늦출 뿐 아니라 궁극적으로는 형질전환 마우스들의 생존 기간을 연장한다는 결론을 얻을 수 있었다. 또한, 각 군당 일정 시기별로 채취된 마우스의 척수 조직을 분석하였을 때 JGK-263 을 투여한 마우스들이 대조군에 비해 운동 신경 세포의 퇴행이 적게 관찰되고 비교적 정상적인 형태를 유지하는 것을 확인할 수 있었다. 각 군당 수집된 마우스의 척수 조직으로 시행한 웨스턴 블롯 실험에서는 JGK-263 을 투여한 마우스에서 세포내 PI3K / Akt 신호 전달 경로가 대조군에 비해 활성화된 것을 확인 할 수 있었고, cleaved caspase-3, cytochrome *c*, PARP-1 등의 세포사멸과 관련된 신호전달 단백질들이 대조군에 비해 유의하게 감소하는 것을 확인할 수 있었다.

결론: 본 연구는 새로운 경구용 GSK-3β 활성 억제 약물인 JGK-263 의 신경보호효과를 NSC-34 운동신경세포주와 SOD1-G93A 형질전환 마우스를 이용한 실험에서 확인할 수 있었다. 이러한 JGK-263 의 신경보호효과는 세포사멸 경로의 억제와 그리고 세포생존 경로의 활성화 기전을 통하여 발현되는 것으로 보이며 향후 근위축성측삭경화증 치료제의 선도적 물질이 될 수 있을 것이다.

주요어 : 근위축성측삭경화증, JGK-263, 세포사멸, GSK-3β억제제

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