

**Enhanced oxidative stress and the treatment by edaravone  
in mice model of amyotrophic lateral sclerosis**

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**Running head:** in vivo imaging of oxidative stress in ALS mice

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## ABSTRACT

Oxidative stress is associated with the degeneration of both motor neurons and skeletal muscles in amyotrophic lateral sclerosis (ALS). A free radical scavenger edaravone has been proven as a therapeutic drug for ALS patients, but the neuroprotective mechanism for the oxidative stress of ALS has not been fully investigated. In this study, we investigated oxidative stress in ALS model mice bearing both oxidative stress sensor nuclear erythroid 2-related factor 2 (Nrf2) and G93A-human Cu/Zn superoxide dismutase (Nrf2/G93A) treated by edaravone. In vivo Nrf2 imaging analysis showed the accelerated oxidative stress both in spinal motor neurons and lower limb muscles of Nrf2/G93A mice according to disease progression in addition to the enhancement of serum oxidative stress marker dROMS. These were significantly alleviated by edaravone treatment accompanied by clinical improvements (rotarod test). The present study suggests that in vivo optical imaging of Nrf2 is useful for detecting oxidative stress in ALS, and edaravone alleviates the degeneration of both motor neurons and muscles related to oxidative stress in ALS patients.

## SIGNIFICANCE

Oxidative stress is associated with the pathological mechanism of ALS. However, anti-oxidative and neuroprotective effects of edaravone, a free radical scavenger, for ALS patients has not been fully investigated. The present in vivo study showed the accelerated expression of oxidative stress marker nrf2 both in spinal motor neurons and lower limb muscles of Nrf2/G93A ALS model mice according to disease progression. Edaravone treatment significantly alleviates both the nrf2 expression and the degeneration related to oxidative stress in both motor neurons and muscles of ALS mice.

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of central and peripheral motor neurons. The majority (90%) of ALS cases are sporadic, while frequent genetic mutations of familial ALS occur in the Cu/Zn superoxide dismutase (SOD1) (Aoki et al., 1993; Rosen, 1993) and TAR DNA binding protein 43 (TDP-43) (Arai et al., 2006; Kabashi et al., 2008), and a hexanucleotide repeat expansion of C9orf72 gene (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Although many pathological mechanisms cause the disease, oxidative stress is associated with motor neuron degeneration in ALS related to SOD1, TDP-43 and the C9orf72 gene (Lopez-Gonzalez et al., 2016; Milani et al., 2013; Moujalled et al., 2017).

The nuclear erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) system is a major oxidative stress sensor; Nrf2 is released from Keap1 in cytoplasm, translocate into nucleus and binds to the antioxidant response element (ARE) under oxidative stress, but Nrf2 combined with Keap1 is rapidly degraded under normal condition (Mead et al., 2013; Petri et al., 2012). We previously reported the Nrf2 activation in spinal motor neurons of ALS model mice bearing G93A-SOD1 (Mimoto et al., 2012), and another report showed a higher ARE activation in skeletal muscles than the spinal cords of the same model mice (Kraft et al., 2007). ALS skeletal muscles are involved in several pathogeneses including oxidative stress (Loeffler et al., 2016; Tsitkanou et al., 2016), but previous reports rarely focused on the contribution of ALS muscles to the pathological process.

For treating oxidative damage in acute ischemic stroke, a free radical scavenger edaravone has been widely used as a neuroprotective drug (Abe et al., 1988; Edaravone Acute Infarction Study, 2003). Recently, edaravone was proven as a therapeutic drug for ALS, improving motor functions of SOD1 mice and rats and ALS patients (Abe et al., 2014; Aoki et al., 2011; Ito et al., 2008; Writing et al., 2017; Yoshino et al., 2006). However, the neuroprotective mechanism of edaravone for the oxidative stress of ALS has not been fully investigated. In the present study,

therefore, we investigated oxidative stress in the spinal cords and skeletal muscles of ALS model mice carrying oxidative stress sensor Nrf2 with in vivo imaging of Nrf2 to observe the possible protective effect of edaravone.

## EXPERIMENTAL PROCEDURES

### Animal models and drug treatments

All experimental procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, Dentistry and Pharmaceutical Sciences of Okayama University (approval #OKU-2015158). Keap1-dependent oxidative stress detector No-48 (Nrf2) (Oikawa et al., 2012) and G93A-human SOD1 (Gurney et al., 1994) transgenic (Tg) mice were obtained from TransGenic Inc. (Fukuoka, Japan) and Jackson Laboratories (Bar Harbor, ME, USA), respectively. Double transgenic mice overexpressing Nrf2 and G93A-SOD1 (Nrf2/G93A) were derived by breeding mice hemizygotously for each Nrf2 transgene with G93A-SOD1 mice. Nrf2/G93A double Tg mice experienced disease onset at around 91 days of age (Ohta et al., 2011), and were randomly divided into two experimental groups: Nrf2/G93A mice treated with vehicle (n = 15; 8 male and 7 female), and Nrf2/G93A Tg mice treated with edaravone (Mitsubishi Tanabe Pharma, Osaka, Japan; n = 14; 6 male and 8 female). Daily intraperitoneal administration of vehicle (saline) or edaravone (15 mg/kg/day) was initiated at 91 days of age, over a period of 28 days (28 injections in total). The two groups were used to assess clinical scores and survival rates, and in vivo optical imaging of oxidative stress along with Nrf2 single Tg mice. The two groups were also analyzed for blood serum, kidney weight and histology along with non-Tg control wild type (WT) B6SJL littermates and Nrf2 single Tg mice.

### Clinical scores and in vivo optical imaging

For clinical analyses, survival was checked every day, and body weight (BW) and the

rotarod score were measured once a week. These analyses were started at 84 days of age (pre-symptomatic stage) in Nrf2/G93A mice treated with vehicle or edaravone according to our previous reports (Ohta et al., 2008; Ohta et al., 2006; Ohta et al., 2016). In the rotarod test, three trials were performed and the best result was recorded. End-stage was defined as the time at which a mouse could not right itself within 30 s when placed on its side.

In vivo bioluminescent imaging was performed in Nrf2 mice and Nrf2/G93A mice treated with vehicle or edaravone using the IVIS spectrum imaging system (PerkinElmer Inc., Billerica, MA, USA). At 84, 105 (early symptomatic stage) and 126 (late stage) days of age (each n=6-12), mice were intraperitoneally injected with 300 mg/kg of D-luciferin (OZ Biosciences, San Diego, CA, USA) dissolved in phosphate-buffered saline (PBS) 15 min before in vivo optical imaging. Under constant anesthesia, the skin of back and lower limbs (LL) was then opened to show the vertebrae and muscles of LL. In the preliminary experiment, mice died too early after laminectomy to detect direct bioluminescent signals from the spinal cords (data not shown). Therefore, we analyzed bioluminescent signals of spinal cords through vertebrae at a 3-min exposure time after incision. In vivo optical signals of oxidative stress in Nrf2/G93A mice were observed as bioluminescence of exogenous human Nrf2 fragment combined with flag-tagged luciferase, induced by endogenous Nrf2 dissociated from Keap1 (Oikawa et al., 2012). Bioluminescent signals of the spinal cords and bilateral LL were measured using regions of interest (ROIs), and emission intensity was expressed as the total flux of photons (photons/s) by LivingImage software (PerkinElmer) (Nakano et al., 2017).

### **Blood serum analyses and kidney weight**

For serum analysis, blood was collected via the retro-orbital puncture from WT, Nrf2, Nrf2/G93A mice treated with vehicle and edaravone at 130 days of age. Serum samples were separated by centrifugation ( $1,940 \times g$ , 15 min, 4°C) and stored at  $-80^{\circ}\text{C}$ . After blood sampling, mice were deeply anesthetized with an intraperitoneal infusion of sodium pentobarbital (10 mg; Abbott Laboratories, Abbott Park, IL, USA), and then perfused with chilled PBS for measurement

of kidney weight and histological analyses.

Serum blood urea nitrogen (BUN) and creatinine (Cr) were measured to analyze renal function in the Department of Animal Resources, Advanced Science Research Center, Okayama University. For the analyses of serum oxidative stress, the reactive oxygen species (ROS) was examined using the d-ROMs test (Diacron International, Grosseto, Italy), and the total serum antioxidant capacity was assayed using the OXY-adsorbent test (Diacron International) according to our previous reports (Kusaki et al., 2017; Tamaki et al., 2011). The serum oxidative stress (OS) index was calculated by dividing the d-ROMs value by the OXY value and multiplying by 1000 (Crowley et al., 2013; Jothery et al., 2016).

### **Immunofluorescence of the quadriceps muscle and spinal cord**

The quadriceps muscles and the lumbar cord spanning L4–L5 were removed from the WT, Nrf2 and Nrf2/G93A mice treated with vehicle or edaravone at 130 days of age, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and placed in PBS containing sucrose 30%. The quadriceps muscles or spinal cords were cut on cryostat in 25- and 10- $\mu$ m sections, respectively.

After blocking with 5% bovine serum albumin (BSA) for 60 min at room temperature, the quadriceps muscle and spinal sections were incubated with primary antibodies at 4°C overnight. Following the overnight incubation, they were incubated again with corresponding secondary antibodies (1:500, Alexa Fluor™, Invitrogen, Carlsbad, CA, USA) or Alexa594 conjugated  $\alpha$ -bungarotoxin (BTX, 1:1,000; Invitrogen, B13423). They were then mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) according to our previous reports (Ohta et al., 2011; Ohta et al., 2016). The following antibodies were used: goat anti-vesicular acetylcholine transporter (VAcHT) (1:500; Millipore, Billerica, MA, USA, ABN100, AB\_2630394), rabbit anti-Nrf2 (1:100; Sigma-Aldrich, St. Louis, MO, USA, SAB4501984, AB\_10747179) interacting with the endogenous mouse Nrf2 but not the exogenous human Nrf2 fragment, goat anti-luciferase (Luc) (1:100; Novus, Littleton, CO, USA, NB100-

1677, AB\_10000585), rabbit anti-peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ ) (1:100; Millipore, AB3242, AB\_2268462), rabbit anti-histone deacetylase 4 (HDAC4) (1:100; Abcam, Cambridge, MA, USA, ab12172, AB\_298904), goat anti-choline acetyltransferase (ChAT) (1:100; Millipore, AB144P, AB\_2079751), mouse anti-neuronal nuclear antigen (NeuN) (1:100; Millipore, MAB377, AB\_2298772), mouse anti-glial fibrillary acidic protein (GFAP) (1:200; Millipore, MAB3402, AB\_10627989), and goat anti-ionized calcium-binding adapter molecule-1 (Iba1) antibodies (1:400, Novus, NB100-1028, AB\_521594) (Table 1). Samples were observed by confocal laser microscopy (LSM780, Zeiss, Oberkochen, Germany).

### **Quantitative analyses of muscle and spinal cord sections**

For the quantitative analysis of myofiber size, or the number of endogenous (stained with anti-Nrf2 antibody) or exogenous Nrf2 (stained with anti-Luc antibody) positive myofibers, about 180 myofibers from three hematoxylin and eosin (HE) stained or immunostained quadriceps muscle sections per mouse (n=5-6 mice for each group) were analyzed by an investigator blinded to the treatment conditions. For the analyses of denervation, 100 neuromuscular junctions (NMJ) from each mouse were analyzed (n = 5-6 mice for each group).

For the analysis of the number of  $\alpha$ -motor neurons stained with both ChAT and NeuN, and endogenous or exogenous Nrf2 positive cells, and the semiquantitative evaluation of immunoreactivity for GFAP and Iba1, four lumbar cord sections from each mouse (n=5-6 mice for each group) were analyzed using ImageJ by an investigator blinded to the treatment conditions.

### **Statistical Analysis**

Data were analyzed in GraphPad Prism (version 6.0, GraphPad Software Inc., San Diego, CA, SCR\_002798) and expressed as means  $\pm$  SD. Statistical comparisons of immunoreactivity for GFAP and Iba1 between Nrf2/SOD1 treated with vehicle and edaravone were performed using

an unpaired t-test. Statistical comparisons of bioluminescent signals of in vivo imaging, kidney weight, blood serum analyses, and other histological data were performed using one-way ANOVA, followed by a Tukey-Kramer post hoc comparison for normally disturbed data, and with Kruskal-Wallis, followed by a Dunn's post hoc comparison for non-normally disturbed data. Statistical comparisons of BW and rotarod score were performed using two-way repeated measure ANOVA. Kaplan-Meier survival analysis and the log-rank test were used for survival. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### Clinical scores in Nrf2/G93A mice

The mean survival time of Nrf2/G93A mice treated with edaravone ( $141.2 \pm 8.9$  days,  $n=13$ , 5 male and 8 female) was almost same as mice treated with vehicle ( $138.9 \pm 8.4$  days,  $n=15$ , 8 male and 7 female) ( $n=28$ ,  $p=0.404$ , Log-rank test, Fig. 1a). There was no sex difference of mean survival time of Nrf2/G93A mice between vehicle and edaravone (male; vehicle  $139.0 \pm 6.9$  days, edaravone  $144.6 \pm 11.8$  days, female; vehicle  $138.9 \pm 8.4$  days, edaravone  $141.2 \pm 8.9$  days). Nrf2/G93A mice treated with vehicle ( $n=11$ , 5 male and 6 female) showed a gradual decrease in body weight after 98 days of age, while those delayed with edaravone ( $n=14$ , 6 male and 8 female) did not experience weight loss (from 91 to 126 days of age:  $F_{1,23}=1.85$ ,  $n=25$ ,  $p=0.187$ , two-way repeated measure ANOVA, Fig. 1b). There was no sex difference of body weight change of Nrf2/G93A mice between vehicle and edaravone (data not shown). Nrf2/G93A mice treated with vehicle ( $n=11$ , 5 male and 6 female) showed a progressive decline in motor performance in the rotarod test from 98 days of age, which was significantly alleviated by edaravone ( $n=12$ , 5 male and 7 female) at 105 days of age (from 91 to 126 days of age:  $F_{1,21}=4.75$ ,  $n=23$ ,  $p=0.041$ , two-way repeated measure ANOVA,  $p<0.05$ , Bonferroni's post hoc test, Fig. 1c). Furthermore, edaravone treatment significantly alleviated the decreased rotarod motor performance at 112 and 119 days of age in male Nrf2/G93A mice (from 91 to 126 days of age:  $F_{1,8}=8.70$ ,  $n=10$ ,  $p=0.019$ ,

two-way repeated measure ANOVA,  $p < 0.05$ , Bonferroni's post hoc test, suppl Fig. 1a), but not in female (from 91 to 126 days of age:  $F_{1, 11} = 0.44$ ,  $n = 13$ ,  $p = 0.520$ , two-way repeated measure ANOVA, suppl Fig. 1b).

### **Mouse serum analyses for renal function and oxidative stress**

The kidney weight of Nrf2/G93A mice treated with vehicle ( $n = 6$ , 1 male and 5 female) and edaravone ( $n = 6$ , 3 male and 3 female) was significantly less than that of WT ( $n = 11$ , 4 male and 7 female) and Nrf2 mice ( $n = 7$ , 3 male and 4 female) ( $F_{3, 26} = 9.58$ ,  $n = 30$ ,  $p < 0.001$ , one-way ANOVA,  $p < 0.01$ , Tukey-Kramer post hoc, Fig. 2a). Serum renal function showed significant elevation of BUN in Nrf2/G93A mice with vehicle ( $n = 5$ , 2 male and 3 female) and edaravone ( $n = 3$ , 3 female) than that in WT ( $n = 10$ , 3 male and 7 female) and Nrf2 mice ( $n = 7$ , 3 male and 4 female) ( $H = 14.16$ ,  $n = 25$ ,  $p = 0.003$ , Kruskal-Wallis,  $p < 0.01$ , Dunn's post hoc, Fig. 2b), while there were no significant differences in the serum Cr level between the four groups (WT;  $n = 10$ , 3 male and 7 female, Nrf2;  $n = 7$ , 3 male and 4 female, vehicle;  $n = 6$ , 3 male and 3 female, edaravone;  $n = 4$ , 1 male and 3 female,  $H = 7.19$ ,  $n = 27$ ,  $p = 0.066$ , Kruskal-Wallis, Fig. 2c). There was no sex difference in kidney weight and serum renal function (data not shown).

The serum markers of oxidative stress dROMs and the OS index were significantly elevated in Nrf2/G93A mice treated with vehicle (vehicle;  $n = 7$ , 2 male and 5 female, edaravone;  $n = 6$ , 3 male and 3 female) compared with WT ( $n = 5$ , 4 male and 1 female) and Nrf2 mice ( $n = 7$ , 3 male and 4 female) (dROMs:  $F_{3, 21} = 6.27$ ,  $n = 25$ ,  $p = 0.003$ , one-way ANOVA,  $p < 0.01$ , Tukey-Kramer post hoc; OS index:  $F_{3, 21} = 3.20$ ,  $n = 25$ ,  $p = 0.044$ , one-way ANOVA,  $p < 0.05$ , Tukey-Kramer post hoc, Fig. 2d, e), which was ameliorated by edaravone. In contrast, there was no significant difference in a serum marker of anti-oxidative activity OXY between four groups ( $F_{3, 21} = 0.37$ ,  $n = 25$ ,  $p = 0.991$ , one-way ANOVA, Fig. 2f). There was no sex difference in dROM, OS index and OXY (data not shown).

### **In vivo optical imaging of oxidative stress of Nrf2/G93A mice**

In Nrf2 mice (84 and 105 days of age; n=6, 4 male and 2 female, 126 days of age; n=7, 5 male and 2 female), an evident signal was not observed over the spine or LL at three time points (Fig. 3a-c). However, weak signals were observed in the LL of Nrf2/G93A mice treated with vehicle at 84 days of age (n=8, 4 male and 4 female,  $F_{2,20}=0.65$ , n=23,  $p=0.531$ , one-way ANOVA, Fig. 3d, j), which were remarkably increased in the spine and LL at 105 days of age, especially in LL of male mice (n=8, 5 male and 3 female, spine:  $F_{2,23}=5.72$ , n=26,  $p=0.010$ , one-way ANOVA,  $p>0.05$  vs Nrf2 mice, Tukey-Kramer post hoc; LL:  $F_{2,23}=12.35$ , n=26,  $p<0.001$ , one-way ANOVA,  $p<0.01$  vs Nrf2 mice, Tukey-Kramer post hoc, male LL:  $F_{2,10}=12.27$ , n=13,  $p=0.002$ , one-way ANOVA,  $p<0.01$  vs Nrf2 mice, Tukey-Kramer post hoc, Fig. 3e, j) with a further increase at 126 days of age (n=8, 5 male and 3 female, spine:  $F_{2,20}=4.07$ , n=23,  $p=0.033$ , one-way ANOVA,  $p>0.05$  vs Nrf2 mice, Tukey-Kramer post hoc; LL:  $F_{2,20}=5.63$ , n=23,  $p=0.012$ , one-way ANOVA,  $p<0.05$  vs Nrf2 mice, Tukey-Kramer post hoc, Fig. 3f, j, arrowheads and arrows). Edaravone treatments, which were initiated at 91 days of age, significantly reduced such signals both in the spine and LL at the early symptomatic and late stages, especially in LL of male mice at the early symptomatic stage (84 days of age; n=9, 3 male and 6 female, 105 days of age; n=12, 4 male and 8 female, 126 days of age; n=8, 1 male and 7 female, spine at 105 days of age:  $p<0.01$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc; spine at 126 days of age:  $p<0.05$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc; LL at 105 days of age:  $p<0.001$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc; male LL at 105 days of age:  $p<0.01$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc; LL at 126 days of age:  $p<0.05$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc, Fig. 3h, i, j).

### **Myofiber size and denervation in quadriceps muscles of Nrf2/G93A mice**

Compared with WT (n=5, 3 male and 2 female) and Nrf2 mice (n=5, 2 male and 3 female), HE staining of quadriceps muscles showed marked or mild neurogenic myofiber atrophy in Nrf2/G93A mice with vehicle (n=6, 1 male and 5 female) and edaravone (n=6, 3 male and 3 female), respectively, at 130 days (Fig. 4a, Supple Fig. 2), when strong bioluminescent signals

appeared in vivo (Fig. 3f). Small myofibers (<20  $\mu\text{m}$  diameter) dominated in Nrf2/G93A mice with vehicle compared with WT and Nrf2 mice (<10  $\mu\text{m}$  diameter:  $F_{3, 18}=18.17$ ,  $n=22$ ,  $p<0.001$ , one-way ANOVA,  $p<0.001$  vs WT and Nrf2 mice, Tukey-Kramer post hoc; 10-20  $\mu\text{m}$  diameter:  $F_{3, 18}=45.49$ ,  $n=22$ ,  $p<0.001$ , one-way ANOVA,  $p<0.001$  vs WT and Nrf2 mice, Tukey-Kramer post hoc, Fig. 4b), which was significantly alleviated by edaravone (10-20  $\mu\text{m}$  diameter:  $p<0.01$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc; 20-30  $\mu\text{m}$  diameter:  $F_{3, 18}=7.01$ ,  $n=22$ ,  $p=0.003$ , one-way ANOVA,  $p<0.05$  vs WT and Nrf2 mice, Tukey-Kramer post hoc, Fig. 4b), showing no sex differences (data not shown). While most NMJ were innervated in WT and Nrf2 mice (Fig. 4c, arrows), detachment of NMJ was extremely high (~90%) both in Nrf2/G93A mice with vehicle and edaravone (Fig. 4c, arrowheads), without sex differences (data not shown).

#### **Oxidative stress markers in quadriceps muscles of Nrf2/G93A mice**

A weak immunoreactivity of endogenous Nrf2 (Endo Nrf2) detected by anti-Nrf2 antibody was observed in quadriceps myofibers of WT and Nrf2 mice, but that of exogenous Nrf2 (Exo Nrf2) detected by anti-Luc was not, except for endomysium (Fig. 5a). In contrast, Nrf2/G93A mice showed strong signals of both Endo and Exo Nrf2 in myofibers of the vehicle treatment group, which was markedly inhibited by edaravone (Fig. 5a, bottom). Nrf2/G93A mice with vehicle ( $n=5$ , 1 male and 4 female) showed an extremely high percentage of both Endo and Exo Nrf2 positive small myofibers (<30  $\mu\text{m}$  diameter), which was significantly reduced by edaravone ( $n=6$ , 3 male and 3 female, <10  $\mu\text{m}$  diameter:  $H=10.19$ ,  $n=22$ ,  $p=0.017$ , Kruskal-Wallis,  $p<0.05$ , Dunn's post hoc; 10-20  $\mu\text{m}$  diameter:  $H=15.09$ ,  $n=22$ ,  $p=0.002$ , Kruskal-Wallis,  $p<0.05$ ,  $p<0.01$ , Dunn's post hoc; 20-30  $\mu\text{m}$  diameter:  $H=13.89$ ,  $n=22$ ,  $p=0.003$ , Kruskal-Wallis,  $p<0.05$ , Dunn's post hoc, Fig. 5b), showing no sex differences (data not shown).

One Nrf2 regulating factor PGC1 $\alpha$  was positive in all myofibers of WT and Nrf2 mice, while another factor HDAC4 was not (Fig. 6a, b). However, both PGC1 $\alpha$  and HDAC4 were positive in Nrf2/G93A mice only in the myofibers expressing Exo Nrf2 (Fig. 6a, b, arrows).

### **Motor neuron degeneration and gliosis in the lumbar cord of Nrf2/G93A mice**

Although the number of  $\alpha$ -motor neurons expressing both ChAT and NeuN was significantly lower in Nrf2/G93A mice with vehicle (n=5, 1 male and 4 female) compared with WT (n=5, 3 male and 2 female) and Nrf2 mice (n=5, 2 male and 3 female,  $F_{3,18}=81.28$ , n=22,  $p<0.001$ , one-way ANOVA,  $p<0.001$  vs WT and Nrf2 mice, Tukey-Kramer post hoc, Fig. 7a, b, Supple Fig. 3, arrows), edaravone treatment significantly ameliorated motor neuron loss in Nrf2/G93A mice (n=6, 3 male and 3 female,  $p<0.001$  vs Nrf2/G93A with vehicle, Tukey-Kramer post hoc, Fig. 7a, b, arrows), showing no sex differences (data not shown). Strong astrogliosis (GFAP) was detected in Nrf2/G93A mice with vehicle, which was significantly decreased by edaravone treatment (n=12, unpaired t-test,  $p=0.040$ , Fig. 7c, d) without sex differences (data not shown). However, microgliosis (Iba1) was not modified by edaravone treatment (n=12, unpaired t-test,  $p=0.694$ , Fig. 7e, f) without sex differences (data not shown).

### **Oxidative stress markers in lumbar cords of Nrf2/G93A mice**

Both Endo and Exo Nrf2 showed no immunofluorescent signal in the lumbar cords of WT and Nrf2 mice (Fig. 8a), while both were expressed in many neuronal cells of only the ventral horns in Nrf2/G93A mice with vehicle (Fig. 8a, arrows), which was significantly reduced by edaravone treatment ( $F_{3,20}=12.44$ , n=24,  $p<0.001$ , one-way ANOVA,  $p<0.05$ ,  $p<0.01$ ,  $p<0.001$ , Tukey-Kramer post hoc, Fig. 8a, b, arrows) showing no sex differences (data not shown). Double immunofluorescent analyses showed that large motor neurons expressed both Endo and Exo Nrf2 (Fig. 8c, d, e, arrows), but astroglia or microglia did not (Fig. 8f, g).

## **DISCUSSION**

In the present study, the elevation of oxidative stress in the spinal cord and LL muscles of symptomatic Nrf2/G93A mice was first shown with in vivo bioluminescent Nrf2 signals (Fig. 3) and with enhancement of serum markers of oxidative stress dROMS and the OS index (Fig. 2d,

e). Immunoreactivities of Endo and Exo Nrf2 in quadriceps myofibers (Fig. 5) and lumbar motor neurons (Fig. 8) were significantly improved by edaravone treatment (Fig. 2d, e, 3, 5, 8) and were accompanied by improvement of motor performance on rotarod test (Fig. 1c).

Oxidative stress is a major neuropathological mechanism for motor neuron degeneration in ALS associated with SOD1, TDP-43 and the C9orf72 gene (Lopez-Gonzalez et al., 2016; Milani et al., 2013; Moujalled et al., 2017). The Nrf2-Keap1 system is the oxidative stress sensor (Mead et al., 2013; Petri et al., 2012), and we previously reported activation of Nrf2 expression in lumbar motor neurons of G93A-SOD1 mice in spinal cord sections. However, we did not examine the muscles (Mimoto et al., 2012). A previous report using the same model mice showed a higher activation of ARE expression in LL muscles than in the spinal cord (Kraft et al., 2007). The in vivo optical imaging used in the present study first showed the in vivo activation of Nrf2 expression both in lumbar motor neurons and LL muscles in Nrf2/G93A mice, which was confirmed by immunofluorescent analyses (Fig. 3, 5, 8). Although previous reports did not pay strong attention to skeletal muscles, the present study showed the enhanced oxidative stress of LL muscles as well as of the spinal cord in ALS model mice (Fig. 3).

ALS skeletal muscles are involved in oxidative stress, mitochondrial dysfunction and bioenergetic disturbances (Loeffler et al., 2016; Tsitkanou et al., 2016). In the present study, Nrf2/G93A mice showed neurogenic myofiber atrophy, denervated NMJ and Nrf2 expression of small quadriceps myofibers in LL, which were significantly alleviated by edaravone, except for the NMJ denervation (Fig. 3-5). This suggests that edaravone directly reduced LL muscle degeneration due to oxidative stress. PGC1 $\alpha$  regulated Nrf2 expression and mitochondrial biogenesis, and improved muscle function in G37R-SOD1 mice (Da Cruz et al., 2012; Do et al., 2014). However, PGC1 $\alpha$  expression was decreased in skeletal muscles both of G93A-SOD1 mice and ALS patients unlike healthy subjects (Thau et al., 2012). Furthermore, another Nrf2 regulating factor HDAC4 was correlated with disease progression in ALS patients (Bruneteau et al., 2013; Singh et al., 2013). In the present study, both PGC1 $\alpha$  and HDAC4 were expressed preferentially in Nrf2 expressing myofibers of Nrf2/SOD1 mice (Fig. 6), suggesting the Nrf2 upregulation for

oxidative stress was independent in skeletal muscles, and the Nrf2 pathway of skeletal muscles can be an alternative therapeutic target in ALS.

A free radical scavenger edaravone was proven as a neuroprotective drug and is widely used for the treatment of acute ischemic strokes (Abe et al., 1988; Edaravone Acute Infarction Study, 2003). Previous reports showed that edaravone reduced the oxidative stress marker 3-nitrotyrosine in the spinal cord of G93A-SOD1 mice and cerebral cerebrospinal fluid of ALS patients (Ito et al., 2008; Yoshino and Kimura, 2006). It also improved motor function of SOD1 mice and rats as well as ALS patients (Abe et al., 2014; Aoki et al., 2011; Ito et al., 2008; Writing and Edaravone, 2017; Yoshino and Kimura, 2006). In the present study, edaravone treatment significantly reduced the oxidative stress serum markers dROM and the OS index, and Nrf2 expression in spinal motor neurons and LL myofibers (Fig. 2d, e, 3, 5, 8). It also reduced the expressions of PGC1 $\alpha$  and HDAC4 in myofibers (Fig. 6), suggesting edaravone treatment enhanced mitochondrial function and muscle development (Loeffler et al., 2016; Petri et al., 2012; Thau et al., 2012). Furthermore, edaravone treatment ameliorated neurogenic myofiber atrophy, spinal motor neuron loss, astrogliosis and motor performance impairment measured by the rotarod test in Nrf2/G93A mice (Fig. 1c, 4, 7). This suggests that the inhibition of oxidative stress is beneficial to reduce degeneration both in the spinal cord and skeletal muscles of ALS. While edaravone treatment did not improve the survival of ALS model mice (Fig. 1a) as previously reported (Aoki et al., 2011; Ito et al., 2008), which suggests that inhibition of oxidative stress is not beneficial to prolong life span in ALS model mice. However, improving motor performance and muscle atrophy due to edaravone treatment is important to preserve activities of daily living for ALS patients (Abe et al., 2014; Writing and Edaravone, 2017). As previously reported (Aoki et al., 2011), edaravone treatment improved the motor performance in male mice, but not in female (Supple Fig. 1), although it ameliorated the oxidative stress and the degeneration of skeletal muscles and spinal cords in both sex (data not shown). The reason might be due to protective effects of estrogen for mitochondria under oxidative stress in female (Cacabelos et al., 2016; Kruman et al., 1999), but further studies using more numbers of each sex are needed to confirm

the sex difference on benefits of edaravone treatment.

In conclusion, we studied accelerated oxidative stress both in spinal motor neurons and LL myofibers of SOD1 mice according to disease progression by serum, in vivo optical and immunofluorescent analyses. Edaravone successfully reduced the degeneration of motor neurons and myofibers by inhibiting oxidative stress. In vivo optical imaging of Nrf2 may be useful for evaluating antioxidative therapies for oxidative stress in ALS patients.

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### **CONFLICT OF INTEREST**

The authors of this study have no conflicts of interest to report.

### **ROLE OF AUTHORS**

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: YO, KA. Acquisition of data: YO, EN, JS, TF, YH, LX, XS, YN, NH, KS, MT, TY. Analysis and interpretation of data: YO, EN. Draft article for important intellectual content: YO, KA. Statistical analysis: YO. Obtained funding and supervised the study: YO, TY, KA.

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## FIGURE LEGENDS

**Figure 1.** Clinical analyses of Nrf2/G93A mice on (a) a Kaplan-Meier cumulative survival curve with vehicle or edaravone, (b) body weight changes, and (c) rotarod scores. Note progressive decline of the rotarod test in Nrf2/G93A mice (c, dotted line), and significant improvement by edaravone (c, solid line) at 105 days of age ( $\star p < 0.05$ ).

**Figure 2.** Renal function (a-c) and serum oxidative stress markers (d-f) in Nrf2/G93A mice. Note (a) kidney weight loss in vehicle (V) and edaravone (E) ( $\#\# p < 0.01$ ) and (b) serum BUN elevation in V and E ( $\#\# p < 0.01$ ). (d, e) Note also the increase of serum oxidative stress markers dROMs (d) and OS index (e) only in V ( $\# p < 0.05$ ,  $\#\# p < 0.01$ ) and not in E.

**Figure 3.** In vivo optical imaging of oxidative stress marker Nrf2 in Nrf2/G93A mice. (a-j) Note strong Nrf2 signals in the spine (e, arrowhead, j) and LL (e, arrows, j,  $\#\# p < 0.01$ ) of vehicle (V) at the early symptomatic stage with a further emphasis at the late stage (f, arrowheads and arrows, j,  $\# p < 0.05$ ), and significant improvement by edaravone (E) (h, i, j,  $\# p < 0.05$ ,  $\#\# p < 0.01$ ,  $\#\#\# p < 0.001$ ).

**Figure 4.** Myofiber atrophy (a, b) and denervation (c) in quadriceps muscles of Nrf2/G93A mice. (a, b) Note significant HE-stained neurogenic myofiber atrophy of quadriceps muscles in vehicle (V), and significant improvement by edaravone (E) (a, b,  $\# p < 0.05$ ,  $\#\# p < 0.01$ ,  $\#\#\# p < 0.001$ ). (c) Note innervated (arrows) NMJs of quadriceps muscles in WT and Nrf2 mice, and denervation (arrowheads) in Nrf2/G93A mice. Scale bar = 50  $\mu\text{m}$  (a), 20  $\mu\text{m}$  (c).

**Figure 5.** Endogenous (Endo) and exogenous (Exo) Nrf2 expression in quadriceps muscles of Nrf2/G93A mice. (a, b) Note both Endo (anti-Nrf2) and Exo (anti-Luc) Nrf2 expression in most small myofibers in vehicle (V), and significant improvement by edaravone (E) ( $\# p < 0.05$ ,  $\#\# p < 0.01$ ). Scale bar = 20  $\mu\text{m}$  (a).

**Figure 6.** Nrf2 regulating factor PGC1 $\alpha$  (a) and HDAC4 (b) expression in quadriceps muscles of Nrf2/G93A mice. (a, b) Note positive signals of both PGC1 $\alpha$  and HDAC4 only in Exo Nrf2 (anti-Luc) expressed myofibers of Nrf2/G93A mice with both vehicle and edaravone (arrows). Scale bar = 20  $\mu$ m (a, b).

**Figure 7.** Motor neuron number loss (a, b) and gliosis (c-f) in the lumbar cords of Nrf2/G93A mice. (a-f) Note a reduced number of  $\alpha$ -motor neurons (MN) (a, anti-ChAT plus anti-NeuN, arrows) and enhanced astrogliosis (c, anti-GFAP) and microgliosis (e, anti-Iba1) in vehicle (V), and significant improvement of MN loss and astrogliosis by edaravone (E) (a, b, <sup>###</sup>p<0.001, c, d, <sup>#</sup>p<0.05). Scale bar = 20  $\mu$ m (a), 50  $\mu$ m (c, e).

**Figure 8.** Endo (anti-Nrf2) and Exo (anti-Luc) Nrf2 expression in lumbar motor neurons of Nrf2/G93A mice. (a, b) Note increased numbers of Endo or Exo Nrf2 positive neuronal cells in vehicle (V), and significant reduction by edaravone (E) (a, arrows show Endo plus Exo positive, arrowheads show only Endo positive, b, <sup>#</sup>p<0.05, <sup>##</sup>p<0.01, <sup>###</sup>p<0.001). (c-g) Note Endo or Exo Nrf2 expression in lumbar large motor neurons (c-e, arrows) of V, but not in astrocyte (f, anti-GFAP) or microglia (g, anti-Iba1). Scale bar = 20  $\mu$ m (a, c-g).

**Supple Figure 1.** Rotarod scores in male (a) and female (b) Nrf2/G93A mice with vehicle (dotted line) and edaravone (solid line). Note significant improvement by edaravone (a, solid line) at 112 and 119 days of age in male mice (<sup>\*</sup>p<0.05), but not in female mice (b).

**Supple Figure 2.** Pictures of HE-stained myofibers in quadriceps muscles of same mice of Fig. 4a at low magnification. Scale bar = 500  $\mu$ m.

**Supple Figure 3.** Pictures of  $\alpha$ -motor neurons (arrows) stained with both anti-ChAT (green) and anti-NeuN (red) in the lumbar cords of same mice of Fig. 7a. Scale bar = 20  $\mu$ m.