

# Effects of epigallocatechin-3-gallate on iron metabolism in spinal cord motor neurons

FENGYUAN CHE<sup>1-3</sup>, GUANGYING WANG<sup>2</sup>, JIXU YU<sup>2,3</sup>, XIANJUN WANG<sup>2</sup>, YUCHENG LU<sup>3</sup>,  
QINGXI FU<sup>2</sup>, QUANPING SU<sup>3</sup>, JIANZHANG JIANG<sup>2</sup> and YIFENG DU<sup>1</sup>

<sup>1</sup>Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021;

<sup>2</sup>Department of Neurology; <sup>3</sup>Central Laboratory, Linyi People's Hospital, Linyi, Shandong 276003, P.R. China

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**Abstract.** Accumulating evidence suggests that iron homeostasis is disordered in amyotrophic lateral sclerosis (ALS). In view of the promising performance of epigallocatechin-3-gallate (EGCG) in neuroprotection studies, the present study aimed to verify whether EGCG protects motor neurons in an ALS model, and whether it has any effects on iron metabolism using an ELISA and western blotting. The results demonstrated that EGCG decreased oxidative stress and protected motor neurons in the organotypic culture of the rat spinal cord. Furthermore, total iron levels increased significantly in the spinal cord following 3 weeks of treatment with threo-hydroxyaspartate. In addition, the expression of influx proteins (transferrin receptor and divalent metal-ion transporter 1) increased significantly. However, EGCG demonstrated no effect on total iron levels and the expression of influx proteins. In conclusion, EGCG leads to a decrease in oxidative stress levels, leading to motor neuron protection in the organotypic culture of a rat spinal cord; however, EGCG does not alter iron metabolism protein expression regulation.

## Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, characterized by the loss of motor neurons in the motor cortex, nucleus of the brainstem and anterior horn of the spinal cord (1). The mechanism of the degeneration of motor neurons in ALS is not well defined and there is no effective therapy that has the ability to block ALS progression. The majority of ALS patients only survive 3-5 years following diagnosis (1).

Accumulating evidence suggests that iron homeostasis is disordered in ALS. Jeong *et al* (2) demonstrated that iron accumulation in the spinal cord of super oxide dismutase (SOD1)<sup>G37R</sup> transgenic mice at 12 months of age and the iron influx proteins [transferrin receptor (TfR) and divalent metal-ion transporter 1 (DMT1)], increased significantly compared with wild-type control mice. Hadzhieva *et al* (3) observed an increased total iron content in G93A-SOD1 SH-SY5Y neuroblastoma cells, and mRNA expression of TfR and DMT1 was increased in G93A-SOD1 cells. In ALS patients, the level of iron in the cerebrospinal fluid was higher than the levels in control subjects (4). In addition, the serum ferritin level was demonstrated to be elevated in patients with ALS (5). Langkammer *et al* (6) identified increased iron deposition in localized regions along the corticospinal tract in ALS patients (6). Corroborating these findings, it was previously reported that phase shift values were significantly higher in the motor cortex of patients with ALS using susceptibility weighted imaging, indicating increased iron levels in this area (7). Additionally, pathological studies have demonstrated increased iron accumulation in the motor cortex of patients with ALS (8).

As it appears that iron levels are affected in ALS, it is possible that drugs regulating iron metabolism may be useful for treatment of ALS. Treatment of SOD1<sup>G37R</sup> transgenic mice with an iron chelator extended the life span by 5 weeks (2). It was also previously reported that epigallocatechin-3-gallate (EGCG) may act as an iron chelator to treat neurodegenerative diseases (9,10). EGCG is the major constituent of green tea polyphenols, accounting for >10% of its composition. EGCG is a natural anticancer agent, and also demonstrated potential neuroprotective functions (11). In previous studies, EGCG has exhibited multifunctional therapeutic effects in a mouse model

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**Correspondence to:** Dr Jixu Yu, Department of Neurology, Linyi People's Hospital, 27 Jiefang Road, Linyi, Shandong 276003, P.R. China  
E-mail: yujixu@yahoo.com

Professor Yifeng Du, Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Weiqi Road, Jinan, Shandong 250021, P.R. China  
E-mail: duyifeng2013@163.com

**Abbreviations:** EGCG, epigallocatechin-3-gallate; ALS, amyotrophic lateral sclerosis; TfR, transferrin receptor; DMT1, divalent metal-ion transporter 1; THA, threo-hydroxyaspartate; MDA, malondialdehyde

**Key words:** epigallocatechin-3-gallate, iron, iron metabolism proteins, motor neuron, organotypic culture, amyotrophic lateral sclerosis, neurodegenerative disease

of ALS (12,13). Thus, the effects of EGCG on iron metabolism were investigated in an ALS model.

## Materials and methods

**Materials.** Threo-hydroxyaspartate (THA) was purchased from Sigma-Aldrich; Merck KGaA (catalog no. H2775; Darmstadt, Germany). The antibody against neurofilament (SMI-32) was purchased from Covance, Inc. (catalog no. SMI-32R; Princeton, NJ, USA) and the antibody against TfR was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (catalog no. 13-6800; Waltham, MA, USA). Antibodies against DMT1 (catalog no. sc-30120) and  $\beta$ -actin (catalog no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

**Organotypic culture of rat spinal cord.** Organotypic spinal cord cultures were prepared as described previously (14-16). Briefly, lumbar spinal cords were removed from 6 to 8-day-old male and female Sprague Dawley rats (Animal Center of Hebei Medical University, Shijiazhuang, China) under sterile conditions and sectioned transversely at 350  $\mu$ m intervals using a tissue chopper (Mickle Laboratory Engineering Co., Ltd., Guildford, UK). The rats were housed in clear plastic cages with sawdust bedding at standard room temperature under a 12 h light/dark cycle. All rats had free access to food and water. Sections were quickly transferred to sterile Gey's balanced salt solution containing glucose (6.4 mg/ml) and separated from one another at room temperature. The tissue slices were placed on the surface of 30 mm Millipore Millicell-CM membranes (EMD Millipore, Billerica, MA, USA), five slices/membrane, and each membrane was then placed in a 33 mm culture well containing 1 ml medium, which consisted of 50% (v/v) minimal essential medium (Gibco; Thermo Fisher Scientific, Inc.) with 25 mM HEPES, 25% (v/v) heat-inactivated horse serum (Gibco; Thermo Fisher Scientific, Inc.) (56°C for 30 min), and 25% (v/v) Hanks' balanced salt solution (supplemented with 25.6 mg/ml D-glucose and 2 mM glutamine). The cultures were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for up to 4 weeks. Culture medium, along with test chemicals, was changed twice per week, unless specified otherwise. THA was dissolved in water. EGCG was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was 0.1% in culture medium. The animal experimental protocol was approved by the Animal Care Committee of Linyi People's Hospital (Linyi, China).

**Treatments on organotypic cultures.** Unless otherwise stated, the spinal cord explants were initially cultured *in vitro* for 7 days. On day 7, the explants were pretreated with 5  $\mu$ M EGCG for 48 h, and then treated with the combination of 5  $\mu$ M EGCG and 100  $\mu$ M THA for 3 weeks. The concentration of 5  $\mu$ M was identified as the effective dose of EGCG on the spinal cord explants in the authors' preliminary experiments (16). The culture medium along with test chemicals was replaced twice per week. At the end of the 3 week treatment, the explants were harvested for analysis.

**Immunohistochemical staining.** Immunohistochemical staining was used to visualize motor neurons in spinal cord

explants. The explants, at the end of an experimental treatment, were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 40 min at room temperature, rinsed three times with 0.1 M PBS, and stored in 0.1 M PBS at 4°C before use. The explants were washed in TBS for 30 min and then treated with 10% horse serum (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The explants were subsequently incubated with the anti-neurofilament antibody (SMI-32: 1:1,000) overnight at 4°C, followed by washing with TBS and Tween-20 three times and incubated with a biotinylated secondary antibody (1:1,000; catalog no. SP-9002; ZSGB-BIO, Beijing, China) for 1 h at room temperature. The explants were further washed and then incubated with a horseradish peroxidase-conjugated ABC staining solution (ZSGB-BIO). The explants were finally mounted on glass slides, and motor neurons in the ventral horns, which were stained dark brown, were counted under a light microscope. This is similar to the previously reported methods (15,16).

**Measurement of malondialdehyde (MDA).** MDA is one of the most important degradation products of lipid peroxidation. It reacts with thiobarbituric acid to produce a product that can be sensitively measured spectroscopically. Following the manufacturer's instructions, enzyme activity was determined by monitoring the change in absorbance at 532 nm using a cell malondialdehyde assay kit from Nanjing Jiancheng Biongingeneering Institute (Nanjing, China).

**Measurement of the total iron.** The total iron content in spinal cord tissue was determined, as described previously (15). The specimens were torrefied at 110°C for 4 h and then examined using a graphite furnace atomic absorption spectrophotometer (AAAnalyst 100; PerkinElmer, Inc., Waltham, MA, USA) by a solid sampling system (SSA 61Z; Analytik Jena AG, Jena, Germany). Absorbance was read at 248.8 nm, and iron content was calculated using a calibration curve prepared with pure iron.

**Western blot analysis.** Spinal cord explants were processed at the end of an experimental treatment to prepare whole tissue extracts, using a tissue extraction reagents kit from Applygen Technologies, Inc. (Beijing, China). The extraction of protein was quantified using a bicinchoninic acid assay. A total of 60  $\mu$ g of extracted protein was resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk for 1 h at room temperature, and then probed with specific primary antibodies [mouse monoclonal anti-TfR (catalog no. 13-6800), 1:500; rabbit polyclonal anti-DMT1 (catalog no. sc-30120), 1:200; mouse monoclonal anti- $\beta$ -actin (catalog no. sc-47778), 1:500] overnight at 4°C, followed with secondary antibodies [goat anti-mouse IgG-horseradish peroxidase (HRP), 1:10,000, catalog no. sc-2005; goat anti-rabbit IgG-HRP, 1:3,000, catalog no. sc-2004 (Santa Cruz Biotechnology, Inc.)] for 1 h at room temperature, and detection was performed with an enhanced chemiluminescence substrate (Beyotime Institute of Biotechnology, Haimen, China). The data were obtained by measuring the density of target protein banding to the density of corresponding  $\beta$ -actin bands, and Quantity One software

version 4.6.7 (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used for quantification.

**Statistical analysis.** Results are expressed as the mean  $\pm$  standard deviation. Statistical analyses were performed using the SAS system (SAS Institute Inc., Cary, NC, USA) for Microsoft Windows version 8 (Microsoft Corporation, Redmond, WA, USA). A one-way analysis of variance followed by the Student-Newman-Keuls test was applied to analyze the data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of EGCG on motor neurons.** After 1 week in culture, rat lumbar spinal cord explants were pretreated with EGCG at 5  $\mu$ M for 48 h and then treated with a combination of EGCG (at the same concentration as pre-treatments) and THA at 100  $\mu$ M for 3 weeks. At the end of the 3 week treatment, the explants were harvested and immunostained with the anti-neurofilament antibody, SMI-32, for the visualization and counting of motor neurons. Representative images of ventral horn neurons of explants treated with vehicle, 100  $\mu$ M THA, and 5  $\mu$ M EGCG plus 100  $\mu$ M THA are presented in Fig. 1A. All motor neurons in both ventral horns of each explant were counted (10 explants/group). The control group averaged  $17.8 \pm 3.42$  motor neurons per explant, but only  $4.8 \pm 0.77$  motor neurons survived per explant following THA treatment (Fig. 1B). This finding is similar to those reported previously, and is significantly decreased when compared with the control group (16). The number of motor neurons ( $19 \pm 3.80$ ) per explant was even higher in explants treated with 5  $\mu$ M EGCG plus 100  $\mu$ M THA when compared with the control group (Fig. 1B), demonstrating that EGCG completely blocked THA-induced motor neuron death.

**Effect of EGCG on lipid peroxidation.** Levels of lipid peroxides were measured in tissue using MDA as a marker of lipid peroxidation. At the end of the 3 week treatment, the spinal cord explants were harvested for measurement of MDA. The concentration of MDA was  $1.07 \pm 0.45$  nmol/mg protein in the control explants, while THA treatment significantly increased the levels of tissue MDA ( $5.35 \pm 2.05$  nmol/mg protein). EGCG prevented the effects of THA and decreased the MDA to  $1.25 \pm 0.54$  nmol/mg protein, which was significantly lower than the THA treated group (Fig. 2).

**Effect of EGCG on total iron.** The total iron content in the explants measured at the end of 3 weeks of THA treatment was increased significantly compared with the control group. However, EGCG did not prevent the iron increase caused by THA. The total iron in the THA-treated group was  $115.27 \pm 18.00$   $\mu$ g/g dry tissue, and  $111.06 \pm 19.35$   $\mu$ g/g dry tissue in the group treated with EGCG plus THA. There was no significant difference between these two experimental groups (Fig. 3;  $P > 0.05$ ).

**Effect of EGCG on iron metabolism protein.** Rat lumbar spinal cord explants were treated with THA and EGCG for 3 weeks and harvested for measurement of expression of iron

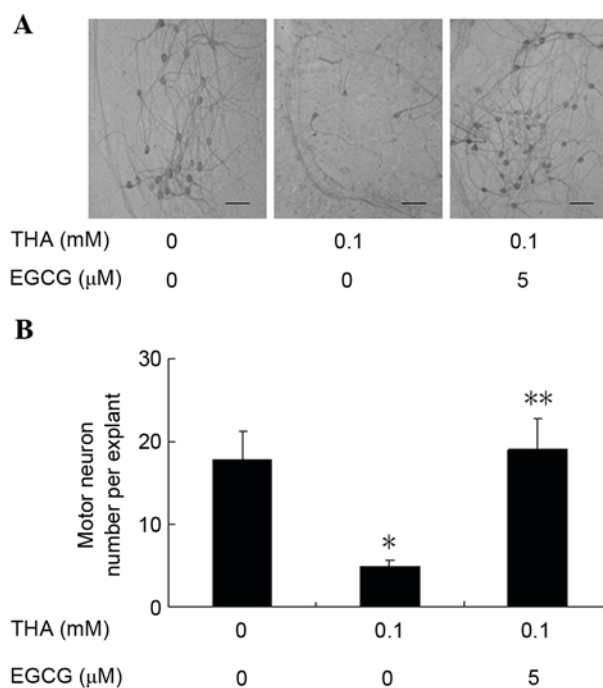


Figure 1. Effect of EGCG on motor neurons in explants of the rat spinal cord. (A) Representative images of immunostaining of explants with SMI-32, presenting motor neurons in the ventral horns of three experimental groups (Scale bar, 100  $\mu$ m). (B) The number of motor neurons per explant in each experimental group (mean  $\pm$  standard deviation,  $n=10$ ). \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. THA only group. EGCG, epigallocatechin-3-gallate; THA, threo-hydroxyaspartate.

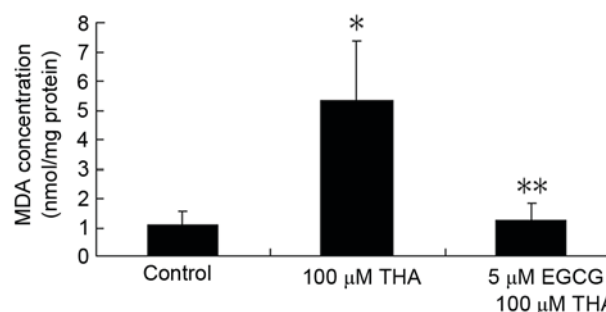


Figure 2. Effect of EGCG on lipid peroxidation. Quantitative assessment of the levels of MDA in three experimental groups (mean  $\pm$  standard deviation,  $n=4$ ). \* $P < 0.05$  vs. control group, \*\* $P < 0.05$  vs. THA only group. EGCG, epigallocatechin-3-gallate; MDA, malondialdehyde; THA, threo-hydroxyaspartate.

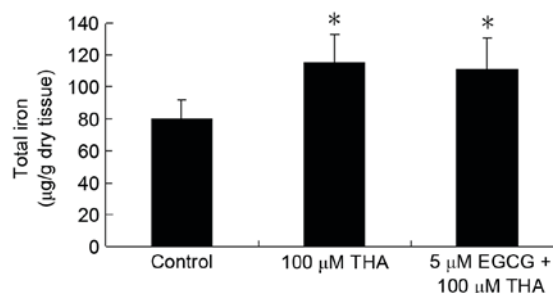


Figure 3. Effect of EGCG on total iron levels. Total tissue iron content was measured by graphite furnace atomic absorption spectrophotometry. \* $P < 0.05$  vs. control group. EGCG, epigallocatechin-3-gallate; THA, threo-hydroxyaspartate.

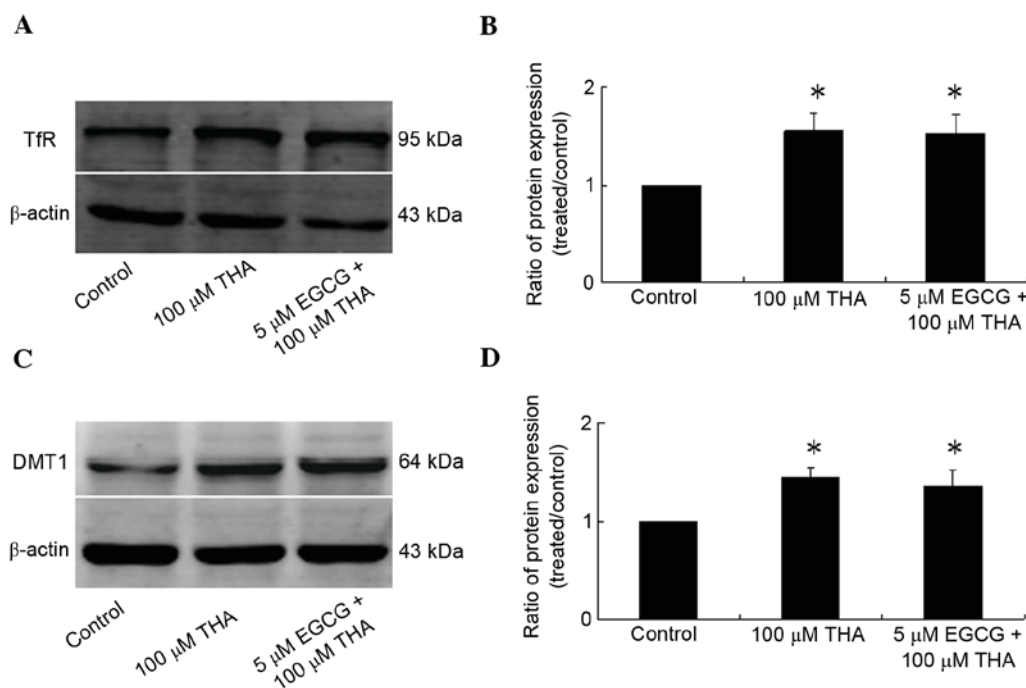


Figure 4. Effect of EGCG on expression of iron metabolism proteins in explants of the rat spinal cord. Densities of (A and B) TfR and (C and D) DMT1 bands were measured and the ratio to  $\beta$ -actin was calculated. The western blotting data are representative of at least three experiments (10-15 explants were pooled in each experiment).  $\beta$ -actin was used as a loading control. Data were expressed as levels of immunoreactivity relative to control values (mean  $\pm$  standard deviation,  $n=4$ ). \* $P<0.05$  vs. control. EGCG, epigallocatechin-3-gallate; TfR, transferrin receptor; THA, threo-hydroxyaspartate; DMT-1, divalent metal-ion transporter.

metabolism proteins. The results demonstrated that the expression levels of TfR and DMT1 increased significantly following THA treatment compared with the control group (Fig. 4; TfR,  $P=0.0316$ ; DMT1,  $P=0.0186$ ). TfR and DMT1 are key cellular iron uptake proteins; however there were no differences in the expression of the TfR and DMT1 between groups treated with EGCG+THA and those treated with THA only (Fig. 4;  $P>0.05$ ), thus EGCG had no effect on the expression levels of TfR and DMT1 in spinal cord explants.

## Discussion

Iron catalyzes the formation of the highly reactive hydroxyl radicals from hydrogen peroxide by the Fenton reaction ( $H_2O_2 + Fe^{2+} \rightarrow OH \cdot + OH^- + Fe^{3+}$ ) and, therefore, it can potentiate the toxic effects of reactive oxidative species (ROS) (17). Previous studies indicate that oxidative stress is a major contributory factor leading to chronic motor neuron death in ALS (18). While iron is essential for normal physiology, the presence of excessive amounts of iron is also implicated in numerous pathological processes, including neurodegenerative disorders (19,20).

THA-induced glutamate excitotoxicity in organotypic spinal cord cultures is a widely used model of motor neuron degeneration that has been used for the development of neuroprotective treatments (21). Glutamate excitotoxicity is thought to result from intracellular calcium overload, leading to the generation of ROS (22). The present study demonstrated that iron levels and the expression of TfR and DMT1 (key cellular iron uptake proteins) increased significantly in spinal cord tissue following THA treatment (15). Therefore, the THA

ALS model was used to study the effect of EGCG on iron metabolism proteins.

In the current study, EGCG (5  $\mu$ M) was demonstrated to protect motor neurons in the organotypic culture of rat spinal cord and decrease lipid peroxidation caused by THA. It appears that EGCG may have a neuroprotective effect at low concentrations. In addition, the total iron content and the expression of influx proteins (TfR and DMT1) increased significantly in spinal cord following 3 weeks of THA treatment. However, there were no differences in total iron levels and the expression of influx proteins (TfR and DMT1) between the groups treated with EGCG+THA and treated with THA only. It appears that, at the dose used, EGCG had no effect on the total iron and the expression of influx proteins in the organotypic spinal cord culture model.

EGCG can protect neurons through many different mechanisms: Scavenging free radicals, chelating transitional metals, modulating the expression of cell survival/death genes and activating phase II drug metabolizing enzymes (23,24). It is possible that EGCG may chelate free iron ( $Fe^{2+}$ ) by removing it from the intracellular iron pool (24-26) and potentially preventing the formation of highly reactive hydroxyl radicals. The present study was unable to measure free iron in the organotypic culture of rat spinal cord due to technical limitations, however there are plans to follow up on the effect of EGCG on cellular free iron in future investigations.

In conclusion, EGCG decreases oxidative stress and protects motor neurons in an organotypic culture of rat spinal cord, but, at the doses given, EGCG could not decrease the influx of iron through regulation of iron metabolism proteins. The study implied that EGCG may not block oxidative damage



caused by iron via chelating iron. Therefore, further studies are required to investigate the mechanism of EGCG protection of motor neurons.

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