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# Folic acid protects motor neurons against the increased homocysteine, inflammation and apoptosis in SOD1$^{G93A}$ transgenic mice

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by selective degeneration of motor neurons. Mutations in copper/zinc superoxide dismutase (SOD1) account for 20% cases of familial ALS (fALS), but the underlying pathogenetic mechanisms are largely unknown. Using SOD1$^{G93A}$ mice model of ALS, we demonstrated that mutation in SOD1 caused a significant increase in the level of plasma homocysteine (Hcy). To investigate whether Hcy-lowering therapy is beneficial to this disease, we applied folic acid (FA) and vitamin B12 which are important factors involved in the Hcy metabolism to assess the neuroprotective effect of FA and B12 in the SOD1$^{G93A}$ mice. Our results showed FA or FA + B12 treatment significantly delayed the disease onset and prolonged the lifespan, accompanied by the significant reduction of motor neuron loss. Furthermore, we found that FA or FA + B12 treatment significantly attenuated the plasma Hcy level, suppressed the activation of microglia and astrocytes, and inhibited the expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) in spinal cord. Moreover, FA or FA + B12 treatment decreased the levels of cleaved caspase-3 and poly(ADP-ribose)polymerase (PARP) but up-regulated the level of anti-apoptotic protein Bcl-2. However, B12 treatment alone did not show any significant benefit to this disease. These results provide evidence to demonstrate that elevated Hcy is involved in the pathogenesis of fALS and FA therapy may have therapeutic potential for the treatment of the disease.



## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequently diagnosed form of adult-onset motor neuron diseases (Cleveland, 1999; Appel, 2006). ALS is characterized by the selective degeneration of lower and upper motor neurons, which lead to progressive weakness and atrophy of skeletal muscles, and eventual paralysis and death (Rowland and Shneider, 2001). Around 10% cases of ALS patients are autosomal dominant, referred as fALS, and 20% patients with fALS have missense mutations in the gene encoding for Cu/Zn superoxide dismutase (SOD1) (Al-Chalabi and Leigh, 2000). Over-expression of mutant SOD1 gene in mice causes a progressive motor neuron disease resembling the most clinical features of the human ALS, which make them suitable for studying the pathogenesis of ALS (Gurney et al., 1994). In this study, we used SOD1$^{G93A}$ mice model to study the association of homocysteine (Hcy) and the development of ALS.

Hcy is a cytotoxic sulfur-containing amino acid, which is produced from methionine by demethylation. The level of Hcy is maintained low by two major mechanisms: one is remethylated to form methionine by folate and vitamin B12, and the other is converted to cystathionine by the activation of the cystathionine-β-synthase (Diaz-Arrastia, 2000). Hyperhomocysteinemia has been reported to be related to Parkinson's disease, Alzheimer's disease and other neurodegenerative disorders (Diaz-Arrastia, 2000; Refsum et al., 1998). Furthermore, it was reported that Hcy-immunoreactive astrocytes presented in the spinal cord of symptomatic SOD1$^{G93A}$ mice and Hcy even at the physiological concentration induced significant cytotoxicity in neuronal cell-line transfected with mutant SOD1 gene, suggesting Hcy may play an important role in the pathogenesis of ALS (Chung et al., 2003; Sung et al., 2002). In addition, high level of Hcy increased the expression of inflammatory factors such as TNF-α and promoted reactive oxygen species (ROS) as well as activated NMDA subtype of the glutamate receptors (Lipton et al., 1997; Refsum et al., 1998; White

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et al., 2001; Wood et al., 2006). Based on these findings, we hypothesize that Hcy may be involved in the pathogenesis of ALS and lowering Hcy may be beneficial to this disease.

FA is a methyl donor in one-carbon metabolism, during which it promotes the remethylation of Hcy (Lucock et al., 1996), while B12 acts as a methionine synthase enzyme (Refsum, 2001). It is suggested that FA and B12 treatment can lower the level of Hcy by remethylation process. In addition, FA is one of the most important elements in the development and adult neural system. Folate deficiency in pregnant women caused an increased risk of neural tube defects in their babies (Blom et al., 2006). Previous study suggested that folate deprivation in SH-SY5Y human neuroblastoma cells induced neurodegenerative changes including increased cytosolic calcium, reactive oxygen species and apoptosis (Ho et al., 2003).

Considering the beneficial effects of FA and B12, we propose that FA and B12 treatment can lower the Hcy level and provide the neuroprotection in SOD1$^{G93A}$ mice. The results of this study may provide the useful information to determine whether FA and B12 treatment has clinical value for the treatment of ALS patients in the future.

## 2. Materials and methods

### 2.1. Animals

The animals used for this study were approved by the Animal Committee of Shanghai Jiaotong University School of Medicine. The colony of well-characterized TgN (SOD1$^{G93A}$) Gur transgenic males, which resemble most clinical features of fALS, were obtained from the Jackson Laboratory (USA) and bred to B6SJL wild-type females. At 1 month of the age, the transgenic offspring were genotyped by PCR assay of DNA obtained from tail tissue.

### 2.2. Experimental protocol

The SOD1$^{G93A}$ mice ($n=48$) were randomized divided into four groups: (1) orally administrated vehicle 0.9% saline (SOD1 group; $n=12$); (2) orally administrated 4 mg folic acid/kg body weight/day (FA-SOD1 group; $n=12$); (3) orally administrated 0.2 mg vitamin B12/kg body weight/day (B12-SOD1 group; $n=12$); and (4) orally administrated 4 mg folic acid and 0.2 mg vitamin B12/kg body weight/day (FA + B12-SOD1 group; $n=12$). All mice at the age of 42 days (6 weeks) started receiving the treatments. Among them, 36 transgenic mice (SOD1 group, $n=9$; FA-SOD1 group, $n=9$; B12-SOD1 group, $n=9$; FA + B12-SOD1 group, $n=9$) were used for the assessment of disease onset and lifespan. Rest of the transgenic mice was used for the assessment of histological and pathological changes. In addition, 10 of the wild-type littermates at the same age as SOD1$^{G93A}$ mice were used as controls for the histopathological staining and biochemical determination.

### 2.3. Behavioral studies

#### 2.3.1. Test of motor function

Rotarod performance was assessed in mice from 70 days of age. Mice were trained for 5 days in order to obtain a baseline. Animals were examined by a blinded observer to determine the age at which the first signs of motor impairment were manifested: the time period that each mouse stayed on a rotating rod (4 cm diameter, 20 rpm) before falling off was less than 5 min (Xu et al., 2006). The rotarod performance for each mouse was tested three times a week and was repeated three times for each test and the longest time of the rotarod performance was used (Andreassen et al., 2001). The date of disease onset was recorded when the mouse stayed on the rotarod less than 5 min.

#### 2.3.2. Assessment of lifespan

The initial sign of disease in SOD1$^{G93A}$ mice is resting tremor and progressive development of gait impairment, asymmetrical, or symmetrical paralysis of the hind limbs followed by complete paralysis at the end stage. The age of death was defined as an animal's inability to right itself within 30 s after being placed on its back (Koh et al., 2007). Animals from all groups were examined three times every week and the date represented the experimental end-point was recorded.

### 2.4. Determination of plasma Hcy level

#### 2.4.1. Sample preparation

Blood samples (150 μL per sample) were obtained from vena caudalis of mice at the age of 120 days. The samples were centrifuged for 10 min at 1500 rpm and plasma were separated and stored at −80 °C until analysis. Reactions were prepared in 1.5 ml microfuge tubes. Both 50 μL of plasma sample with internal standard (D8-Homocystine, IS, 5 μmol/L) and dithiothreitol (300 mmol/L) were added and mixed. The mixture was placed under room temperature for 30 min, and then 50 μL of trichloroacetic acid (15%) was added and followed by centrifugation for 3 min at 16,000 rpm. Supernatant (5 μL) was injected into LC–MS/MS system for analysis.

#### 2.4.2. LC–MS/MS analysis

The Shimadzu LC20AD system (Kyoto, Japan), equipped with two pumps, a vacuum degasser, an auto-sampler and a controller module, were used for this assay. Chromatographic separation was performed on Thermo Aquasil $C_{18}$ column (50 mm × 2.0 mm, 5 μm, Thermo, USA) at room temperature. The mobile phase was constituted of methanol and water, both containing 0.02% formic acid, for isocratic elution with a flow rate of 0.2 ml/min. The auto-sampler was kept at 4 °C and 5 μL samples were injected.

The MS/MS system was a triple quadrupole mass spectrometer API 3000 instrument (ABI-SCIEX, Toronto, Canada) equipped with Turbo Ionspray source and operated in positive ionization mode. Analyst 1.4 software package was used for instrument control and data acquisition. The ion spray voltage was set at 2.5 kV and source temperature at 450 °C. The collision activated dissociation was set at 12 using nitrogen as collision gas. Hcy and internal standard were detected with transitions of 136/90 amu and 140/94 amu, respectively.

### 2.5. Histopathological analysis of spinal cord

SOD1$^{G93A}$ mice (SOD1 group, $n=3$; FA-SOD1 mice, $n=3$; B12-SOD1 group, $n=3$; FA + B12-SOD1 group, $n=3$) and wild-type mice ($n=3$) at the age of 120 days were anesthetized with chloral hydrate and sacrificed by trans-cardiac perfusion with phosphate buffered saline (PBS) at pH 7.4 for 10 min. For histopathological analysis, the spinal cord ($L_{4-5}$) was removed, postfixed overnight in 4% paraformaldehyde and subsequently dehydration in 30% sucrose for 48 h. For western blot assay, the spinal cord (C1–L3) was quickly removed and preserved in liquid nitrogen for further analysis.

Lumbar spinal cord ($L_{4-5}$) were embedded in optimal cutting temperature compound and frozen at −80 °C. Serial transverse sections (10 μm thickness) of the lumbar segment ($L_{4-5}$) were cut and mounted on gelatin coated slides. Serial sections (200 slices) were stained with cresyl violet for Nissl staining, and then sections were dehydrated in a graded alcohol series, cleared in xylene and covered by glass slide. Sections were photographed under the light microscopy. We counted two sides of the anterior horn on every third section between the L4 and the L5 levels of spinal cord of all the four group mice (Kieran et al., 2004). An examiner who was blinded to the experimental design counted the anterior horn cells that met all of the following criteria: (1) neurons located in the anterior horn ventral to the line tangential to the ventral tip of the central canal; (2) neurons with a maximum diameter of 20 μm or more; and (3) neurons with a distinct nucleolus (Manabe et al., 2003; Warita et al., 1999).

### 2.6. Immunohistochemical evaluation of lumbar spinal cord

The sections were treated with 1% $H_2O_2$ for 2 min in order to block the endogenous peroxidase and then rinsed in PBS containing 0.3% Triton. After incubation for 1 h with 10% normal bovine serum in PBS–0.3% Triton, the sections were reacted with a primary antibody overnight at 4 °C. We used the primary antibody of glial fibrillary acidic protein (GFAP, 1:2000, Sigma, St. Louis, MO, USA) and CD11b (1:100, Serotec, Oxford, UK) to examine the activation of astrocyte and microglia, respectively. The sections were then washed three times for 10 min each to remove unbound primary antibodies and incubated with the secondary antibodies conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO, USA) and cy3, respectively. Unbounded secondary antibodies were removed by three rinses of 10 min each. The immunohistochemical-positive cells on the slides were visualized under microscope ×400 (Olympus, USA). Negative controls were performed by incubating sections with secondary antibodies only. We observed the different status of astrocyte and microglia according to their morphological appearance in the anterior horn of spinal cord (L5).

### 2.7. Western blot analysis

Snap-frozen spinal cords were dissolved in RIPA lysis buffer (50 mM Tris–HCL pH 7.4, 1% NP-40, 0.25%Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1 μg/ml Pepstatin) by ultrasonic crasher. Protein (40 μg) of each sample was separated in 8% SDS gel, transferred onto 0.45 μm PVDF membrane, incubated with primary antibodies of iNOS (1:5000, Chemicon, CA, USA), TNF-α (1:1000, Cell signaling, MA, USA), Bcl-2 (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz), cleaved caspase-3 (1:1000, Cell signaling, MA, USA), PARP and cleavage (1:1000, Cell signaling, MA, USA), respectively. After incubation overnight, the blot was washed and then incubated with peroxidase-conjugated secondary antibodies of goat anti-rabbit IgG (Pierce, IL, USA), or goat anti-mouse IgG (Pierce, IL, USA), and then protein bands were visualized using ECL (Pierce, IL, USA). Afterwards the blots were stripped and stained with beta-actin antibody (1:4000, Cell signaling, MA, USA). The results of Western blots were quantified with an image analyzer to measure the density of each interested band (Bio-Rad, Quantity One-4.2.0).

### 2.8. Statistics

Disease onset and survival analysis was performed by Kaplan–Meier analysis (SPSS 13.0), which was analyzed using the log-rank test and generates a $\chi^2$ value to test for significance. Other data were analyzed with the test of ANOVA, and differences with $P$ values less than 0.05 were considered statistically significant. Data are expressed as mean ± SEM.

## 3. Result

### 3.1. Effects of FA and B12 on the onset of symptoms and lifespan in SOD1$^{G93A}$ mice

To explore whether FA or B12 treatment can influence the onset of symptoms in SOD1$^{G93A}$ mice, we analyzed the motor function of all animals by conducting rotarod test. SOD1$^{G93A}$ mice usually recapitulated the clinical progression of ALS by displaying overt hind-limb disability at the age about 100 days and the animals usually died around 130 days. We found the mice in FA-SOD1 group or FA + B12-SOD1 group showed significant delay of the onset of symptoms compared with the mice in SOD1 group (114.4 ± 1.7, 116.3 ± 2.0 vs 107.9 ± 1.8 days, $\chi^2 = 6.33$ and $\chi^2 = 8.989$, respectively, $P < 0.05$). However, no significant delay was found in B12-SOD1 group mice (109.4 ± 1.7 vs 107.9 ± 1.8 days, $\chi^2 = 0.015$, $P > 0.05$) (Fig. 1A).

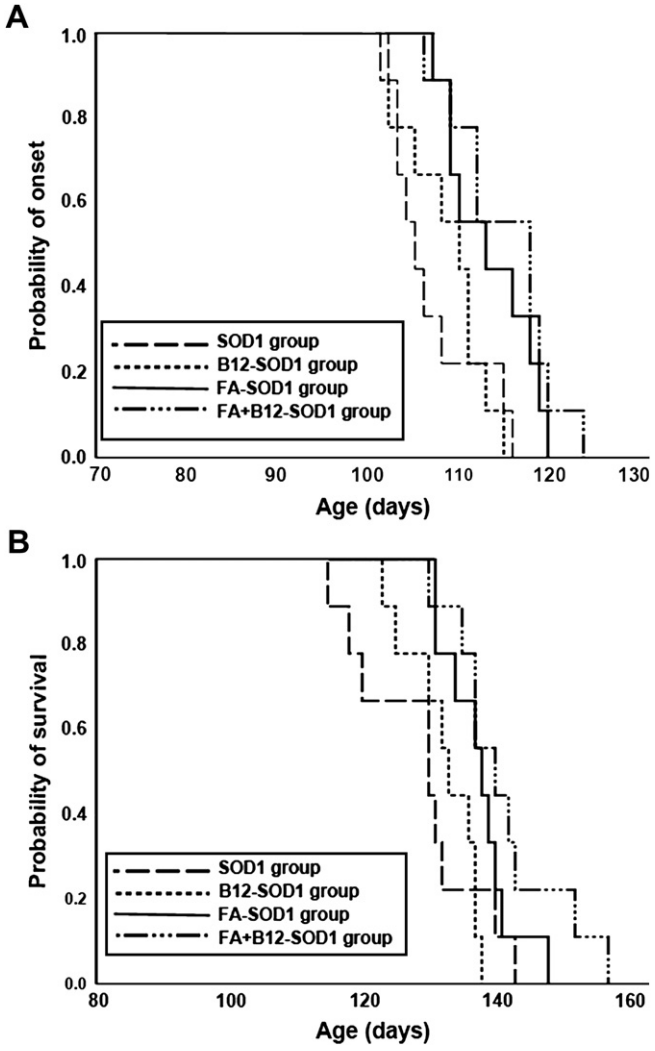


**Fig. 1.** Effects of FA and B12 on the disease onset and the lifespan of SOD1$^{G93A}$ mice. The results of Kaplan–Meier survival analysis (SPSS 13.0) demonstrate the probability of disease onset (A) and the probability of survival (B) in the SOD1 group, B12-SOD1 group, FA-SOD1 group and FA + B12-SOD1 group. *$N$ in each group = 9.

In addition, FA or FA + B12 treated SOD1$^{G93A}$ mice had significant 9-day and 13-day extension in lifespan compared with SOD1 group mice, respectively (137.7 ± 1.9, 141.4 ± 2.9 vs 128.8 ± 3.4 days, $\chi^2 = 3.95$ and $\chi^2 = 5.0$, respectively, $P < 0.05$). There was no significant difference between B12-SOD1 group and SOD1 group in the lifespan (132.3 ± 1.9 vs 128.8 ± 3.4 days, $\chi^2 = 0.015$, $P > 0.05$) (Fig. 1B).

### 3.2. Effects of FA and B12 on the level of plasma Hcy

LC–MS/MS was performed to examine the total plasma Hcy level in SOD1$^{G93A}$ and wild-type litter mate mice at the age of 120 days (eight mice in each group). The level of Hcy in SOD1 group was 180% of the level in wild-type mice (6.84 ± 0.4 vs 3.8 ± 0.26 μmol/L); while, in FA and FA + B12 treatment groups, the level of Hcy was attenuated by 61% and 69%, respectively (4.98 ± 0.38, 4.75 ± 0.67 vs 6.84 ± 0.4 μmol/L in SOD1 group mice) (Fig. 2). Surprisingly, B12 treatment showed a slight but insignificant reduction in the level of Hcy (6.56 ± 0.8 μmol/L vs 6.84 ± 0.4 μmol/L in SOD1 group mice) (Fig. 2). These results provided substantial evidence that FA or FA + B12 treatment but not B12 alone could significantly reduce the level of Hcy in the SOD1$^{G93A}$ transgenic mice.

### 3.3. Effects of FA and B12 on motor neurons survival

To determine the effect of FA or B12 on the motor neurons survival, we used Nissl staining to investigate the morphology of motor neurons in the ventral horn of the lumber spinal cord ($L_{4-5}$). In SOD1 group mice at the age of 120 days, there were much fewer motor neurons compared with wild-type mice (219.67 ± 21.32 vs 792 ± 40.07; $n = 3$; $P < 0.001$) (Fig. 3A, a and b). In FA-SOD1 group or FA + B12-SOD1 group, there were 74.5% and 88.3% more motor neurons than mice in SOD1 group (383.5 ± 24.43, 413.67 ± 32.48 vs 219.67 ± 21.32; $n = 3$; $P < 0.01$) (Fig. 3A, d and e). However, there was no significant change in the motor neurons survival after B12 administration alone (248 ± 24.49 vs 219.67 ± 21.32; $n = 3$; $P > 0.05$) (Fig. 3A, c). Morphometric evaluation of the surviving motor neurons showed that FA alone or combined use of FA and B12 could rescue a significant number of motor neurons according to the result of Nissl staining (Fig. 3B).

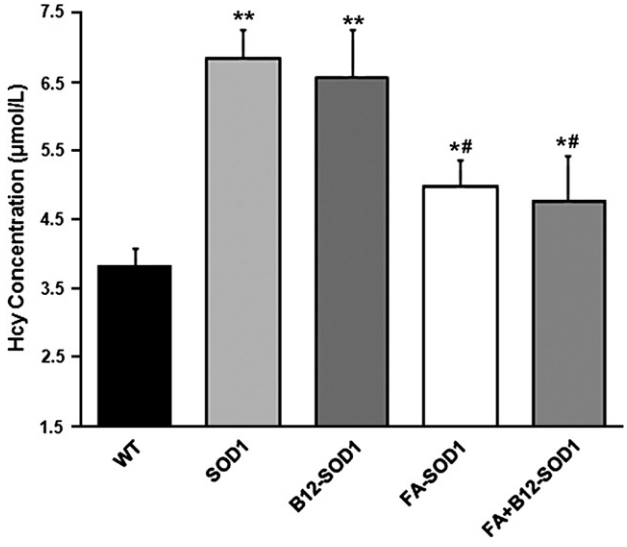


**Fig. 2.** Hcy assay. Plasma Hcy level was measured by LC–MS/MS at the age of 120 days of five group mice. Data showed that the level of Hcy in SOD1 group was increased as compared with the wild-type mice. Hcy level of FA-SOD1 or FA + B12-SOD1 group mice was significantly decreased as compared with SOD1 group mice. Data are mean ± SEM. Analysis of variance was analyzed by ANOVA. *$P < 0.01$ and **$P < 0.001$ (when compared with the wild-type group); #$P < 0.01$ (when compared with the SOD1 group); *$N$ in each group = 8.

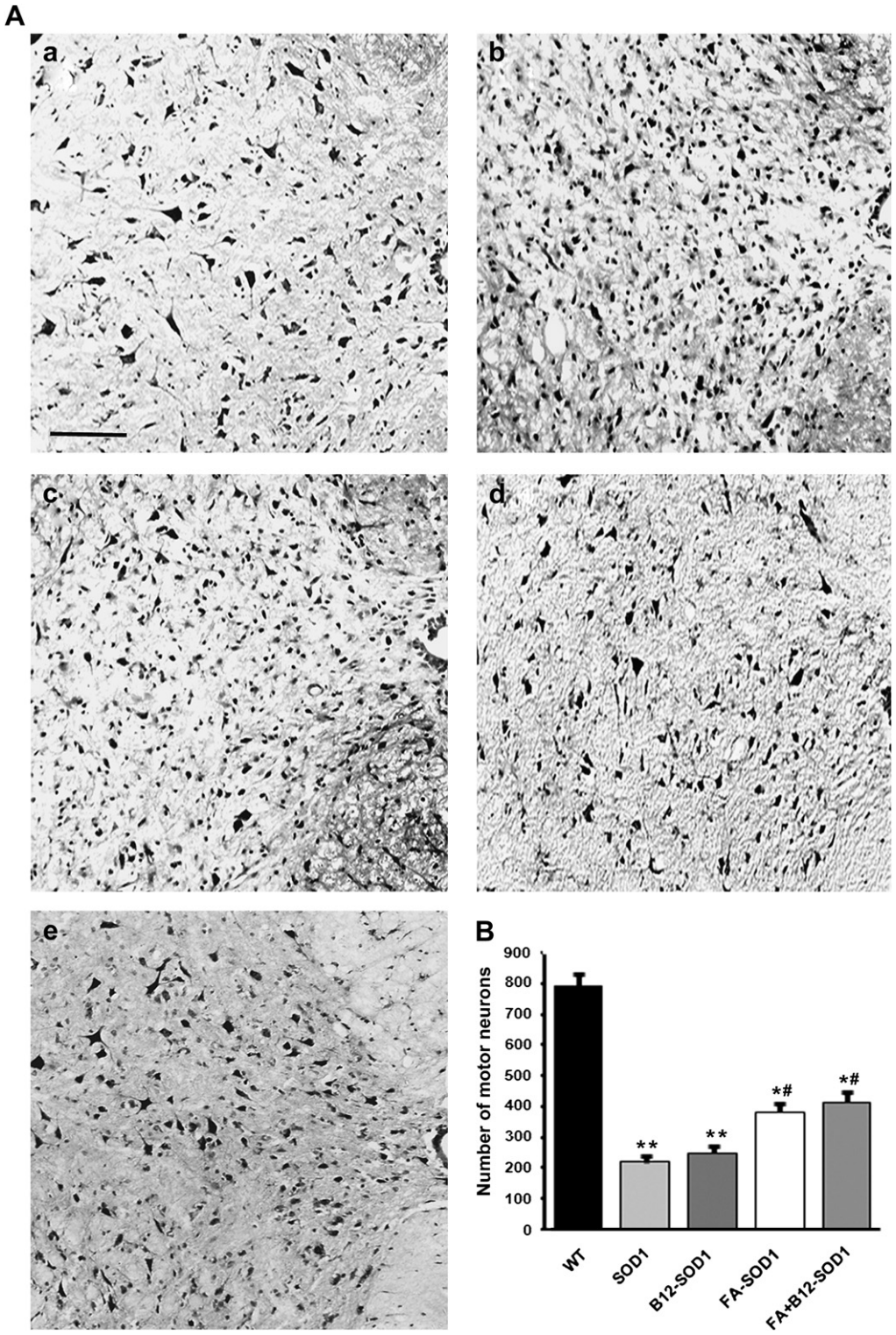


**Fig. 3.** Effects of FA and B12 on the motor neurons survival at the age of 120 days of G93A transgenic mice. (A) Representative photomicrographs of motor neurons in the anterior horn of spinal cord of five group mice, respectively. (a) Wild-type group (b) SOD1 group (c) B12-SOD1 group (d) FA-SOD1 group (e) FA + B12-SOD1 group. (B) The number of motor neurons in $L_{4-5}$ segments. The values are mean ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.001$ (when compared with the wild-type group); $^{\#}P < 0.01$ (when compared with the SOD1 group); $^{*}N$ in each group = 3. Scale bar = 100 μm.

### *3.4. Effects of FA and B12 on activation of glial cells and inflammation-related factors*

Inflammation plays an important role in the pathogenesis of ALS. Glial activation is one of the important markers for the inflammation in the transgenic mice. In our study, we used GFAP and CD11b to examine the status of microglia and astrocyte, respectively, in the spinal cord sections of $SOD1^{G93A}$ mice. We found overwhelming microglial and astrocytic activation in the spinal cord of $SOD1^{G93A}$ mice at the age of 120 days, whereas the mice treated with FA especially with FA + B12 showed significant suppression in microglial and astrocytic activation (Fig. 4). However, there was no significant

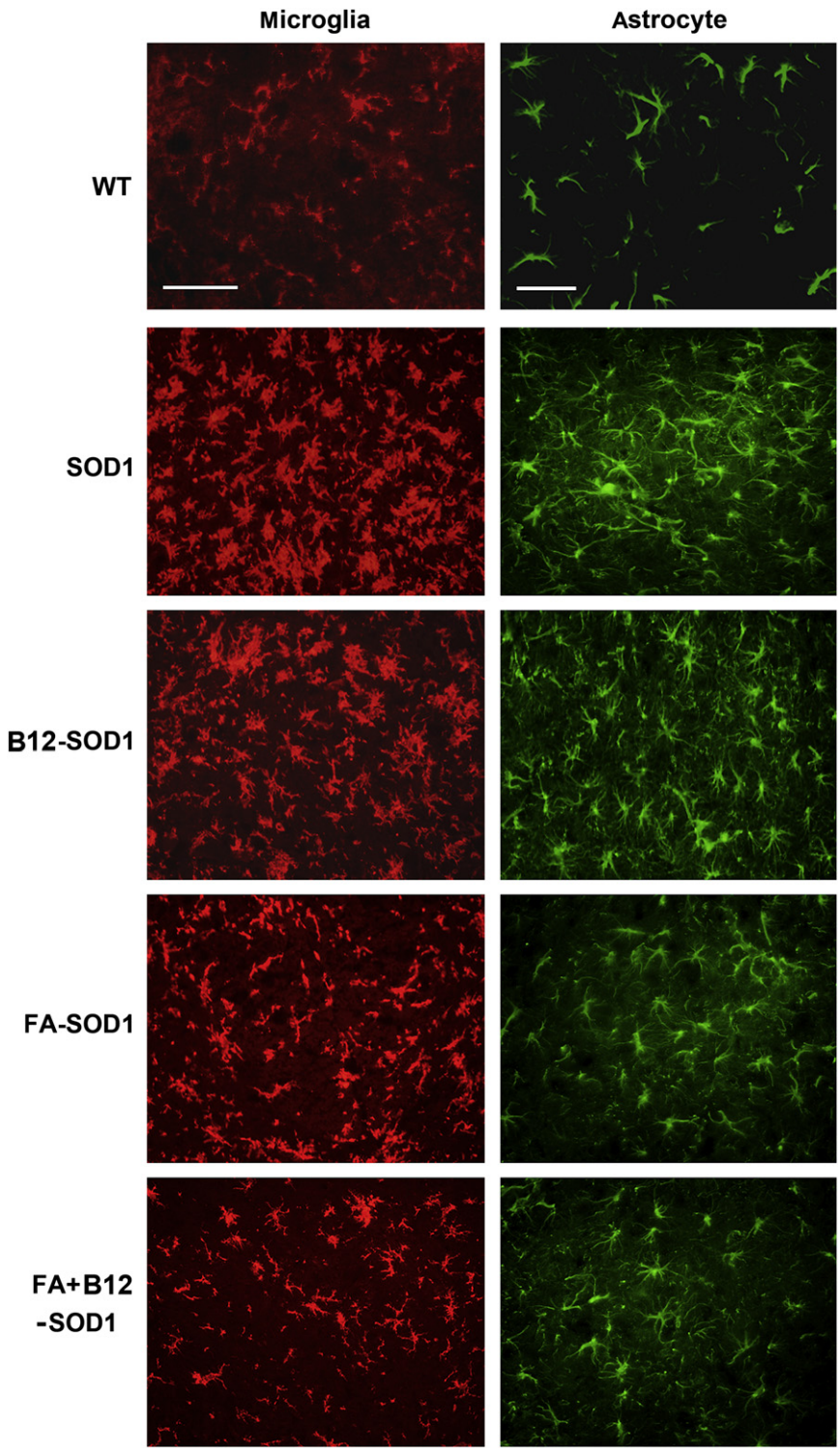


**Fig. 4.** Effects of FA and B12 on the activation of glia. CD11b and GFAP were used as the markers of microglia (red) and astrocyte (green), respectively. The results of immunohistochemical staining revealed that the microglial and astrocytic activation was significantly suppressed in FA-SOD1 or FA + B12-SOD1 group mice as compared with SOD1 group mice. While there was no difference between B12-SOD1 group mice and SOD1 group mice. *$N$ in each group = 3. Scale bar = 30 μm.

difference of glial activation between the mice treated with B12 alone and control $SOD1^{G93A}$ mice (Fig. 4).

It was reported that there was significant increase in the expression of inflammation-related factors such as iNOS and TNF-α in $SOD1^{G93A}$ transgenic mice (Almer et al., 1999; Hensley et al., 2002; Xu et al., 2006). Therefore, in our study, we examined the protein levels of iNOS and TNF-α in the spinal cord of the mice. We observed significant reduction of iNOS and TNF-α in FA-SOD1 group or FA + SOD1 group mice compared with mice in SOD1 group (Fig. 5A). In addition, we found FA + B12 was more effective in lowing the level of iNOS (Fig. 5C). These results documented that the inflammation in

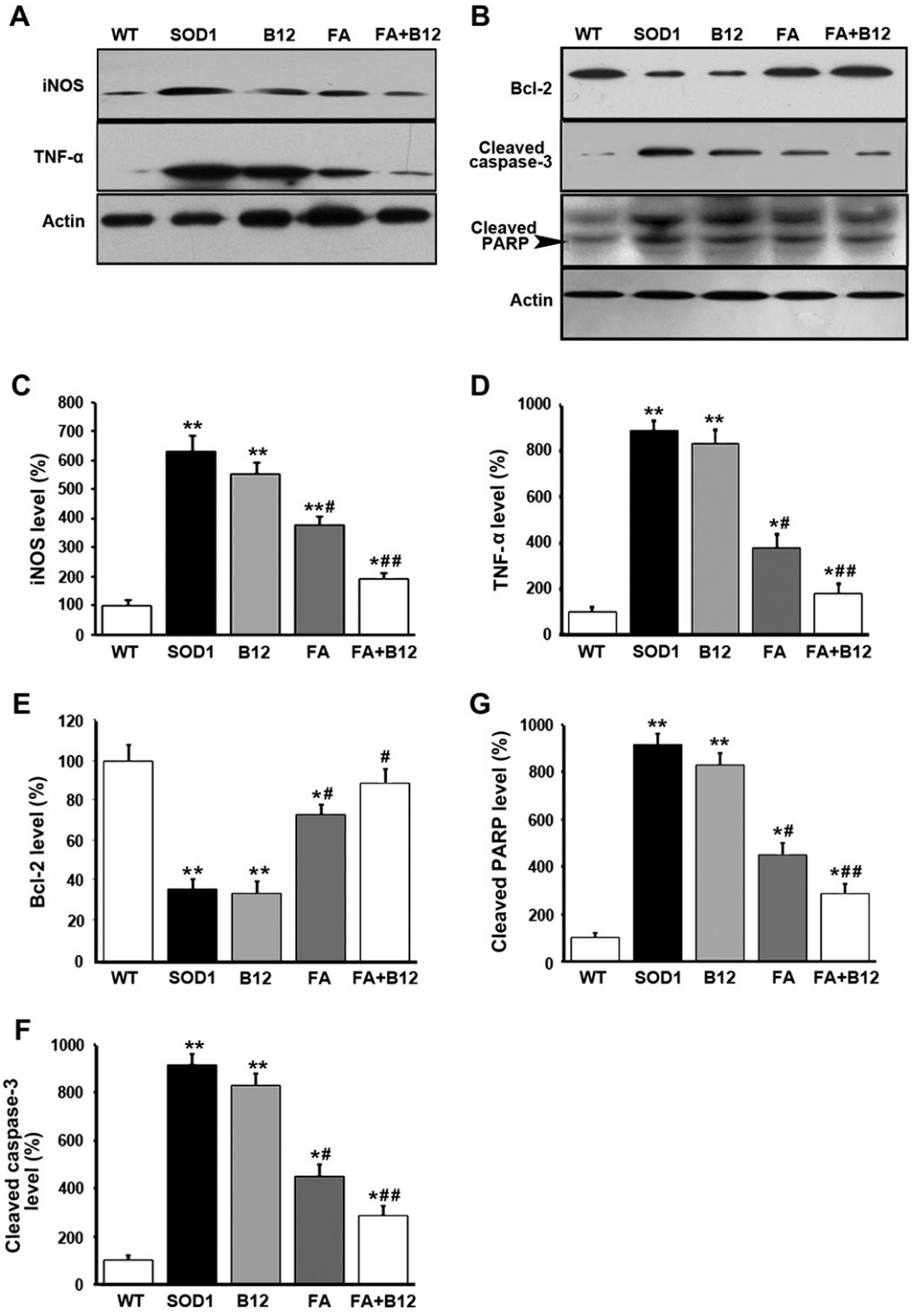


**Fig. 5.** Effects of FA and B12 on the expression of inflammation- or apoptosis-related factors. (A) Western blot of iNOS and TNF-α from spinal cord samples of G93A transgenic mice in five groups (WT: wild-type group; SOD1: SOD1 group; B12: B12-SOD1 group; FA: FA-SOD1 group; FA + B12: FA + B12-SOD1 group). (B) Western blot of Bcl-2, cleaved caspase-3 and cleaved PARP from spinal cord samples. (C–G) Quantitative data of the expression of iNOS, TNF-α, Bcl-2, cleaved caspase-3 and cleaved PARP in five groups. The values are mean ± SEM. $^{*}P < 0.01$ and $^{**}P <$ 0.001 (when compared with the WT group); $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ (when compared with the SOD1 group); $^{*}N$ in each group = 3.

FA-SOD1 group or FA + SOD1 group was strongly suppressed compared with SOD1 group.

### 3.5. Effects of FA and B12 on the expression of apoptosis-related molecules

Previous studies reported that high level of Hcy could trigger neurons death (Kruman et al., 2000). To investigate whether Hcy-lowering drugs FA and B12 had the anti-apoptotic effects in the $SOD1^{G93A}$ transgenic mice, we determined the protein level of Bcl-2, cleaved caspase-3 and PARP in the spinal cord after FA, B12 or FA + B12 treatment. We found that FA or FA + B12 treatment can increase the level of Bcl-2 and reduce the level of cleaved caspase-3 and cleaved PARP (Fig. 5B). Especially, we found FA + B12 treatment can elevate the level of Bcl-2 in $SOD1^{G93A}$ mice up to the level of wild-type, which could suggest that FA + B12 is effective in

suppressing apoptosis in the SOD1 mice model. However, there was no significant change of these apoptosis-related molecules in the mice of B12-SOD1 group (Fig. 5B). The data revealed that the FA or FA + B12 treatment displayed anti-apoptotic effects through suppressing these apoptosis-related molecules.

## 4. Discussion

Our study provided first evidence that FA or FA + B12 treatment can delay the onset of disease and prolong the lifespan by protecting motor neurons against apoptosis in ALS transgenic mice. The neuroprotective effects of FA or FA + B12 treatment in ALS transgenic model may be related to their biological role in Hcy metabolisms since the FA or FA + B12 treatment significantly attenuates the increased level of Hcy in SOD1$^{G93A}$ mice. Furthermore, our study showed that FA or FA + B12 treatment can suppress the production of inflammatory factors such as iNOS and TNF-α, and inhibit the activation of microglia and astrocytes. Moreover, we demonstrated that FA or FA + B12 treatment has significant anti-apoptotic effects by increasing Bcl-2 expression and inhibiting cleaved caspase-3 and cleaved PARP.

Although several studies have showed that there might be association between Hcy and ALS, the role of Hcy in ALS development is not well defined. Hcy is known to exert a pro-inflammatory effect by elevating the level of cytokine, which may contribute to the progression of ALS (Holven et al., 2006). It is suggested that Hcy can enhance pro-inflammatory cytokines production such as interleukin-6, TNF-α and C-reactive protein (Holven et al., 2006). Bai et al. (2007) reported that Hcy could increase the expression of TNF-α, which plays a critical role in inflammatory responses and apoptosis. Furthermore, TNF-α can switch resting murine astrocytes to active state, and up-regulate iNOS expression and subsequently release nitric oxide (Falsig et al., 2004). In addition, it was reported that the expression of iNOS was increased in the spinal cord of the SOD1$^{G93A}$ transgenic mice, and activated microglia and astrocytes increased the production of iNOS, which suggest that iNOS may contribute to the pathology of ALS and represent a valuable therapeutic target for the disease (Almer et al., 1999; Park et al., 2007). In our study, we found FA or FA + B12 treatment could decrease the level of Hcy and suppressed the glial activation as well as the expression of iNOS and TNF-α in the spinal cord of SOD1$^{G93A}$ mice at the age of 120 days. Our results indicate that alteration in Hcy may play a role in the increased inflammation of SOD1$^{G93A}$ mice model, and lowering the level of Hcy by FA or FA + B12 treatment can relieve the inflammation via suppressing the inflammatory factors expression and glial activation.

Accumulating evidence supports that the eventual process of motor neuron death in ALS may result from the activation of apoptotic pathways, especially the caspase-dependent pathway. Hcy could induce apoptosis by regulating the expression of several important proteins such as Bcl-2, cleaved caspase-3 and cleaved PARP (Baydas et al., 2005; Kruman et al., 2000). Bcl-2 plays a prominent role in ALS pathogenesis, which is involved in the oxidative stress and in the mitochondria mediated apoptosis (Pasinelli et al., 2004). Bcl-2 family is implicated in the regulation of motor neuron death in SOD1$^{G93A}$ transgenic mice model and over-expression of Bcl-2 can significantly protect motor neurons in SOD1$^{G93A}$ mice (Kostic et al., 1997). In our study, we found that FA or FA + B12 treatment can increase the expression of Bcl-2, which provided an evidence for the neuroprotective effect of FA or FA + B12 treatment in SOD1$^{G93A}$ mice. Cleaved caspase-3 and cleaved PARP represent the downstream signals of apoptosis, which may contribute to the motor neuron death in SOD1$^{G93A}$ mice (Pasinelli et al., 2000). In addition, reactive astrocytes could express cleaved PRAP in the central nervous system in SOD1$^{G93A}$ mice (Chung et al., 2004). Furthermore, Kruman et al. (2000) reported that Hcy could induce apoptosis in neuronal cells resulting from the activation of PRAP. Our study suggested that FA or FA + B12 treatment could up-regulate the expression of anti-apoptotic protein Bcl-2 as well as down-regulated the expression of apoptosis-related proteins such as cleaved caspase-3 and cleaved PARP. These results highlighted the finding that the decrease in the level of Hcy through the treatment of FA or FA + B12 could provide anti-apoptotic effect in the ALS transgenic mice.

In B12 treated group, we found no significant change in the level of Hcy, inflammation and apoptosis in the SOD1$^{G93A}$ transgenic mice. One possible explanation is that B12 is less effective than FA in reducing the Hcy level in the Hcy-methionine cycle since B12 only acts as a cofactor while FA provides the methyl group to Hcy (Carmel et al., 2003); as a result, inflammation and apoptosis remain high in B12 treated SOD1$^{G93A}$ transgenic mice.

In summary, we documented that FA or FA + B12 treatment delayed the onset of disease and prolonged the lifespan accompanied by the attenuation of motor neuron loss in ALS transgenic mice. Furthermore, we demonstrated that FA or FA + B12 possessed anti-inflammatory effects through inhibiting the expression of iNOS and TNF-α and suppressing the activation of microglia and astrocytes. Moreover, we found that FA or FA + B12 treatment can significantly inhibit the levels of cleaved caspase-3 and cleaved PARP as well as up-regulate the levels of Bcl-2. Collectively, our results indicate the therapeutic potential of FA or FA + B12 for the treatment of ALS.

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