Short Communication: Neuroprotective Effect of Spirulina in a Mouse Model of ALS

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Abstract: Nutritional approaches to the treatment of aging and neurodegenerative diseases have been shown to have potential benefits. Fruits or vegetables provide a cocktail of phytochemicals with multiple actions. Spirulina, a blue green alga used for thousands of years as a food source by the Aztecs, is known to contain large amounts of β-carotene and several phycocyanins with potent antioxidant and anti-inflammatory effects. We examined neuroprotective effects of a spirulina 0.1% supplemented diet in the G93A SOD1 mouse model of ALS beginning at 5 weeks of age and continuing for 10 weeks. Spirulina dietary supplement significantly maintained body weight and extension reflex, and reduced inflammatory markers and motor neuron degeneration in G93A mice. These findings provide initial evidence that nutrition supplementation with spirulina has a neuroprotective support for dying motor neurons. A spirulina supplemented diet may be a potential alternative or adjunctive treatment for ALS.

Keywords: G93A SOD1 mice, diet, spirulina, neuroprotection.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative motor neuron disease that clinically manifests as progressive muscular weakness leading to paralysis and death within three to five years of diagnosis. Most available treatments for this disease are palliative, unable to arrest disease progression or repair motor neurons. The sporadic ALS (SALS) form dominates; with only 5-10% of ALS cases being familial (FALS) or genetically linked. Clinical presentation and underlying pathology of SALS and FALS are similar.

Evidence for oxidative stress has been found in both forms of ALS, tempting one to consider the possibility that similar mechanisms are at play in disease pathogenesis [1, 2]. Moreover, since missense mutations in SOD1, the main free radical scavenging enzyme that protects cells against oxidative stress, have been found in FALS [3, 4], oxidative damage may play a crucial role in ALS. One theory is that mutations in SOD1 lead to a decreased affinity for Zn and this tips the body's cellular balance towards apoptosis and that peroxynitrite is an intermediate [5]. Peroxynitrite is a strong oxidizing agent that has been suggested as a common link in many neurodegenerative diseases and is also an important mediator of inflammation [6]. In the brainstem and spinal cord of both ALS patients and animal models, increased numbers of activated microglia and astrocytes accompanied by IgG deposits and infiltration of T lymphocytes were observed [7-10]. The increases of macrophage-secreted cytokines such as interleukin (IL) 1α, IL 1β and IL 1RA [11], the pro-inflammatory enzyme cyclooxygenase type 2 (Cox-2) [8, 12, 13], and up-regulation of the tumor necrosis factor-alpha (TNF-α) gene [11, 14] make them likely candidates for the effectors damaging motor neurons. These cytokine increases preceded bulk changes in protein oxidation and apoptosis, suggesting that the inflammatory reaction is a primary event precipitating oxidation and apoptosis of motor neurons.

Our studies and others have shown the potential beneficial actions of nutritional approaches to the treatment of aging and neurodegenerative diseases [15-19]. Fruits or vegetables provide a cocktail of phytochemicals with multiple actions including antioxidant and anti-inflammatory effects. For example, blueberries are known to contain numerous phenolic compounds such as anthocyanins that are potent antioxidants; the phenolic content varies in different berry varieties [20]. Adding Gingko biloba extract (EGb761), which has antioxidant properties as a free radical scavenger, to the diet of G93A SOD1 mice significantly improved motor performance and mouse survival [21]. Spirulina, a blue green alga used for thousands of years as a

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food source by the Aztecs, is known to contain large amounts of β-carotene [22] and several phycocyanins [23], all with potent antioxidant effects. Phycocyanin is also known to have potent COX-2 inhibitory actions [24, 25], actions that may be important as COX-2 is increased in ALS [13]. Foods containing phycocyanin may have dual benefits. We have shown potent decreases in markers of oxidative damage and inflammation in aged rats fed diets supplemented with spirulina or spinach [15, 26]. Moreover, spirulina, blueberries, and spinach can reduce ischemic damage following middle cerebral artery occlusion in rats [19], suggesting that nutritional approaches may provide a broad spectrum of neuroprotective actions in a model of neurodegeneration. In the present study, we examined neuroprotective effects of a spirulina supplemented diet in the G93A SOD1 mouse model of ALS.

Transgenic male mice B6SJL-TgN (SOD1-G93A) 1GUR (G93A; obtained from Jackson Laboratories, Bar Harbor, MA, USA) over-expressing human SOD1, carrying the Gly93→Ala mutation, were used. Fifteen G93A mice randomly assigned to one of two groups received a regular control (NIH 31) diet (n=7) or a diet supplemented with spirulina 0.1% (n=8) beginning at 5 weeks of age and continuing for 10 weeks. The control NIH 31 diet was a typical basal diet. The spirulina diet consisted of the basal diet supplemented with 0.1% spirulina powder. Mice were housed individually in cages. Food (diets) and water were available *ad libitum*. All described procedures were approved by the Institutional Animal Care and Use Committee at USF and conducted in compliance with the *Guide for the Care and Use of Laboratory Animals*. Data are presented as means ± S.E.M and were analyzed by Mann-Whitney test. GraphPad InStat (*GraphPad Software, Inc.*) software was used.

CHARACTERISTICS OF DISEASE PROGRESSION

Evaluation of animal disease progression was as previously described [27-29]. All measures of disease progression were performed blind by independent investigators to avoid subjective bias. At 5 weeks of age (before beginning the diets) and then weekly until 15 weeks of age, body weights of mice were measured. Results demonstrate that G93A mice receiving the spirulina 0.1% dietary supplement significantly maintained body weights compared to G93A mice with control diet (Fig. **1A**). Significant differences
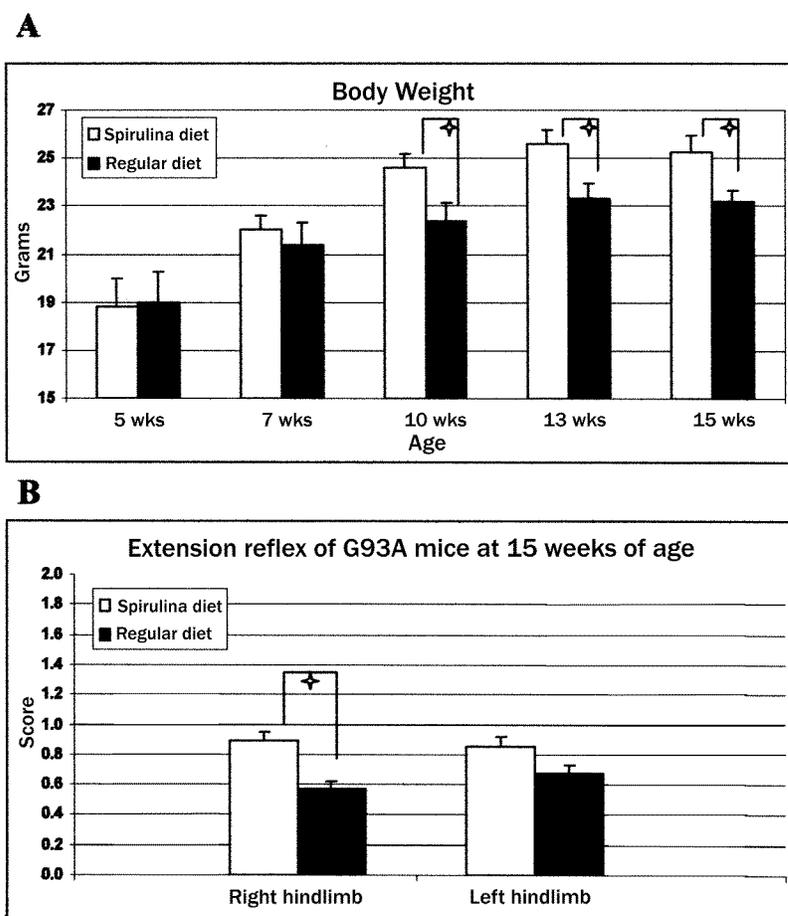
Fig. (1). Body weight and extension reflex of G93A mice with spirulina dietary supplementation. (**A**) G93A mice receiving the spirulina 0.1% dietary supplement starting at 5 weeks of age and continuing for the 10 following weeks significantly maintained body weights compared to G93A mice on the control diet. Significant differences (p<0.05) in body weights between mice fed with spirulina or control diets were observed beginning at 10 weeks of age. (**B**) Extension reflex of hindlimbs of G93A mice measured at 15 weeks of age showed a significant (*p*<0.05) effect of spirulina diet. Mice receiving spirulina for 10 weeks retained extension reflex of right hindlimbs *vs*. mice on the control diet. *p*<0.05.

(p<0.05) in body weights between mice fed with spirulina or control diets were observed beginning at 10 weeks of age, 5 weeks after beginning supplementation. At 15 weeks of age, body weights of G93A mice on the control diet were 23.2±0.44 g *vs*. 25.3±0.69 g for mice on spirulina supplementation. Although a control mice group was not included in this study, data from our previous published work [29, 30], showed that control mice such as transgenic mice (BL6/SJL) carrying the normal allele for SOD1 gene typically maintained their body weights of ~30 g at this age. G93A mice carrying the mutant SOD1 gene usually begin experiencing decreased body weight at 13 weeks of age, when first disease symptoms appear, due to muscle atrophy. Extension reflex of each hindlimb was determined at 15 weeks of age, prior to euthanatasia, when each mouse was suspended by the tail. If the mouse showed normal hindlimb extension, a score of 2 was given. A score of 1 indicated partial hindlimb extension. If no extension was observed, the score was 0. Mice receiving spirulina for 10 weeks retained extension reflex *vs*. mice on the regular diet (Fig. **1B**). Interestingly, extension of right hindlimbs in mice fed the spirulina diet was significantly (p<0.05) higher vs. control diet mice. Although, extension of left highlimbs of these mice tended to be sustained, no significant differences were found between the two groups of mice. Numerous reports [31-33], including ours [27-30], have shown that disease symptoms become apparent at approximately 90 days of age (13 weeks of age) and motor deficits have been detected as early as 8 weeks of age [34] in G93A mice. In our study, G93A mice fed with spirulina for 10 weeks showed significant differences in maintaining body weight and

extension reflex at 15 weeks of age vs. mice on the control diet. Similar results were obtained when oral administration of green Gingko biloba extract (EGb761) was added to the diet of G93A SOD1 mice [21], significantly improving motor performance and survival of these mice.

RIBONUCLEASE PROTECTION ASSAY

Mice were sacrificed under deep pentobarbital (100 mg/kg, ip) anesthesia at 15 weeks of age. The brainstem, cervical and lumbar spinal cord segments were removed from five mice in each group, frozen in liquid nitrogen, and stored at -80°C. Ribonuclease Protection Assay was performed to determine cytokine profiles in the brainstem and spinal cord of mice as previously described [15]. Total RNA from homogenized tissues was extracted using Qiagen Rneasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, twenty μg of total RNA from each sample was hybridized with antisense, radio-labeled probes, after which the free probe and remaining single-stranded RNA were digested with RNase A/T1. The probe (Pharmingen) included mouse specific sequences for interleukin-1 α (IL-1 α), IL-1 β IL-6, and tumor necrosis factor α(TNF α). The phosphorimaging screen was subsequently scanned with a phosphorimager (Molecular Image System GS-363; Bio-Rad). The images were processed using Molecular Analyst software (Bio-Rad). The intensity of a band in the computer generated image was directly proportional to the amount of radioactivity within the band. The optical density (OD) values obtained from each band were normalized against the OD obtained from the L32 band
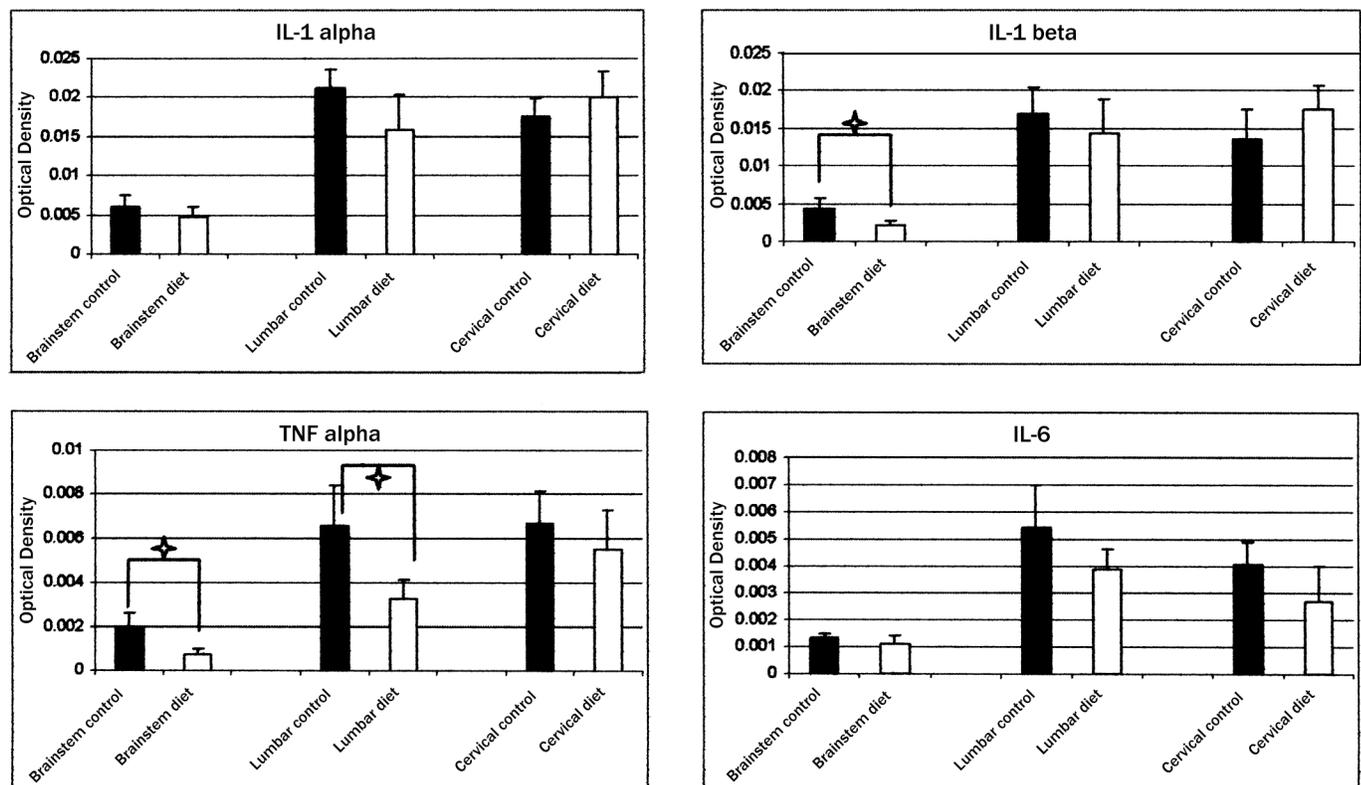

Fig. (2). Characteristics of pro-inflammatory cytokine expression in the brainstem and spinal cords of spirulina fed and control diet G93A mice at 15 weeks of age. Spirulina diet significantly (p<0.05) decreased IL-1 beta in the brainstem and TNF alpha in the brain and lumbar spinal cord. *p*<0.05.

in that sample by the following expression: OD of the sample band / OD of the L32 band X 100. Significant ($p<0.05$) decreases of IL-1 beta (brainstem) and TNF alpha (brainstem and lumbar spinal cord) were detected in spirulina-diet animals vs. control mice (Fig. **2**). In the cervical spinal cord, there were no significant differences for studied cytokines between mice fed with spirulina or regular diet, however, a tendency towards decreasing TNF alpha and IL-6 in mice receiving spirulina was noted.

IMMUNOHISTOCHEMISTRY

For immunohistochemical studies, the remaining mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2). The lumbar segments of the spinal cord were removed, post-fixed, and then cryoprotected in 20% sucrose in 0.1 M PB overnight. Coronal sections (30 μm) were cut in a cryostat, thaw-mounted on slides, and stored at -20°C. The lumbar spinal cords were double stained for Fluoro-Jade, a marker of degenerating neurons, and glial fibrillary acidic protein (GFAP), an astrocyte marker. The 0.001% Fluoro-Jade solution was used according to the manufacturer's protocol. After

staining, the sections were incubated in a blocking solution and then polyclonal rabbit anti-GFAP antibody (1:500, Dako) was applied and the slides were incubated overnight at 4°C. The slides were rinsed and incubated with goat-anti-rabbit secondary antibody (1:700, Alexa Fluor 594) conjugated to rhodamine. After several rinses, the slides were coverslipped with Vectashield (Vector Laboratories) and examined under an epifluorescence Olympus BX60 research microscope. In controls, the primary antibody was omitted. Double immunohistochemical staining for GFAP and Fluoro-Jade of the lumbar spinal cords demonstrated numerous activated astrocytes and neural degeneration in control fed mice at 15 weeks of age (Fig. **3a**). Large degenerated motor neurons were detected in these mice (Fig. **3b**). In G93A mice fed with spirulina supplement, consistent qualitative decreases of activated astrocytes and neural degeneration in the lumbar spinal cord were noted at the same age (Fig. **3c**). Some motor neurons were partially positive for Fluoro-Jade indicating the process of degeneration (Fig. **3d**).

In summary, our study results present initial evidence that nutrition supplementation with spirulina may provide neuroprotective support for dying motor neurons in a mouse model of ALS. Our results showed that the spirulina dietary
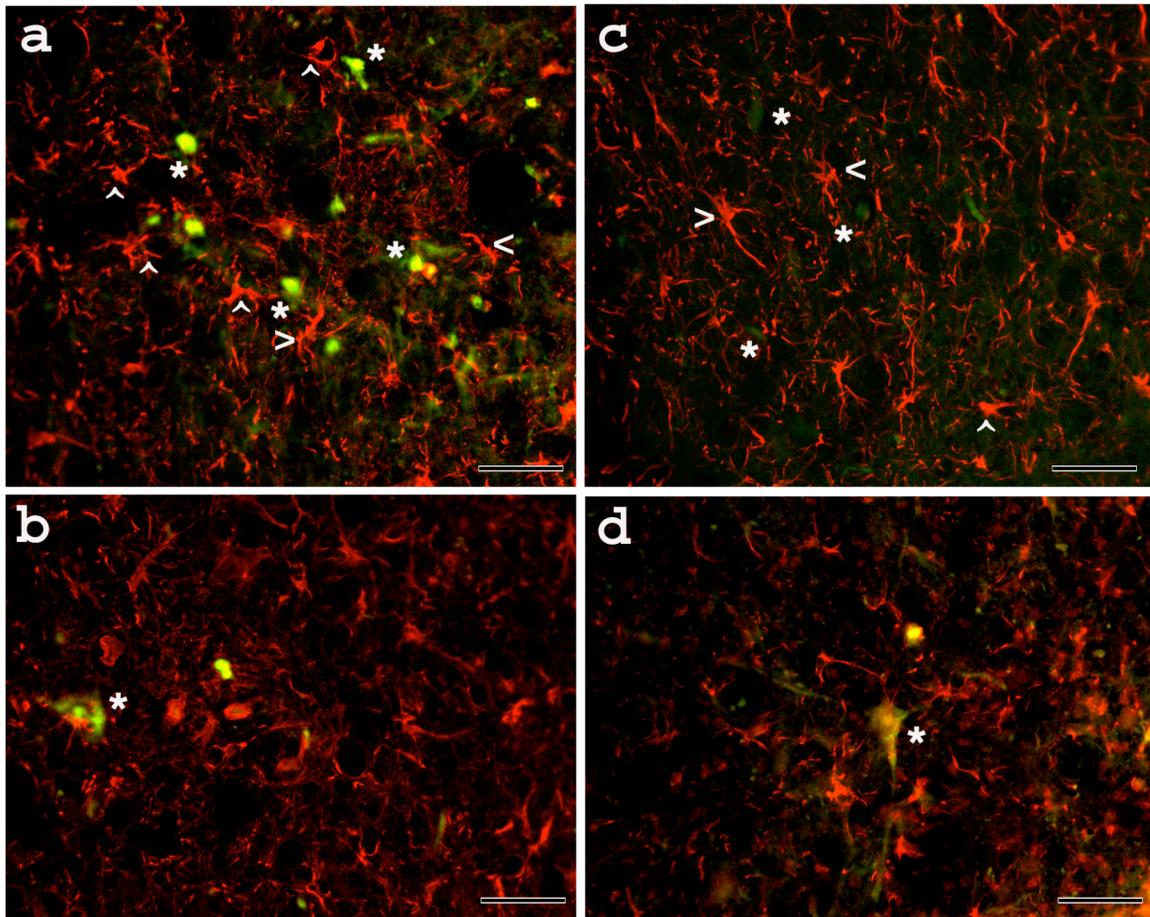
Fig. (3). Immunohistochemical staining for astrocytes (GFAP) and neural degeneration (Fluoro-Jade) in the lumbar spinal cord of G93A mice at 15 weeks of age. (**a**) Astrocyte activation (red, arrowheads) and neuronal degeneration (green, asterisks) in ventral horn of the lumbar spinal cord were observed in control diet G93A mice. (**b**) Degenerated large motor neurons (green, asterisk) were seen in control fed mice at 15 weeks of age. (**c**) Fewer activated astrocytes (red, arrowheads) and degenerated (green, asterisks) neurons were found in spirulina fed mice. (**d**) In these mice, some large motor neurons were beginning to degenerate (yellow, asterisk). Scale bar in a-d is 50 μm.

supplement slowed the onset of motor symptoms and disease progression, reducing inflammatory markers and motor neuron death in G93A mice. Indications were that a reduction of inflammatory processes for preventing motor neuron degeneration might be achieved with the spirulina diet. Green Gingko biloba is considered to have protective effects against mitochondrial damage and oxidative stress [21], yet spirulina may have dual, antioxidant and anti-inflammatory, effects on motor neurons. Although motor neuron counts were not performed in this study, the quantitative analysis of motor neurons survival is important. This question and others will be addressed in our future experiments. Investigation of different concentrations of spirulina dietary supplement will be necessary to find the most beneficial nutritional approach. Also, determining the effect of a spirulina supplemented diet on lifespan of G93A mice is essential to prove the efficacy of our proposed treatment. Thus, a spirulina supplemented diet may have future clinical benefit in treating ALS as an alternative or adjunctive therapy.

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CONFLICT OF INTEREST

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