DIETARY SUPPLEMENTATION WITH PECANS DELAYS MOTOR NEURON PATHOLOGY IN TRANSGENIC MICE EXPRESSING G93A MUTANT HUMAN SUPEROXIDE DISMUTASE-1

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ABSTRACT: A growing body of evidence indicates that diet can modulate health in aging to the extent of delaying the manifestation of age-related diseases. Nuts are among the antioxidant-rich foods that have been demonstrated to provide a degree of protection against age-related disorders. We examined herein whether or not dietary supplementation with pecans could affect the course of pathology in a mouse model of the age-related human motor neuron disorder amyotrophic lateral sclerosis (ALS). Transgenic mice expressing the G93A mutation of human superoxide dismutase-1 SOD-1 have been widely utilized to study the onset and progression of familial ALS. Mice provided a diet supplemented with 0.05% pecans displayed a significant delay in decline in motor neuron function, which was accompanied by increased survival of motor neurons and a decrease in reactive gliosis, as compared to non-supplemented mice. These findings support inclusion of pecans and/or other nuts as part of a comprehensive nutritional therapeutic approach that may augment pharmacological approaches.

KEY WORDS: Amyotrophic Lateral Sclerosis, Gliosis, Motor Neuron Disease, Mouse Model, Neuromuscular Performance, Nutrition, Super Oxide Dismutase-1

INTRODUCTION
A growing body of evidence indicates that diet can modulate health in aging to the extent of delaying the manifestation of age-related diseases (Chernoff, 2001; Everitt et al., 2006; McKeith, 2005; Shatenstein, 2008). Evidence has been presented that consumption of nuts can alleviate the risk and/or severity of cardiovascular disease (Blomhoff et al., 2006; Kelly and Sabate, 2006; Nash and Nash, 2008; Sabate and Fraser, 1994; Salas-Salvado et al., 2006) and diabetes (Jenkins et al., 2008; Lovejoy, 2005). Less is known regarding potential beneficial effects to age-related trauma of the nervous system. Constituents of nuts known to be beneficial in cardiovascular disease, including alphatocopherol, phenolics, and reservatol (Griel and Kris-Etherton, 2006; Kris-Etherton et al., 2008; Miraliakbari and Shahidi, 2008; Ryan et al., 2006) also provide protection against disorders of the central nervous system when consumed as supplements or in other foods (Calabrese et al., 2007; Duffy et al., 2008; Foley and White, 2002; Galli et al., 2006; Joseph et al., 2007; Joseph et al., 2003; Joseph et al., 1999; Lau et al., 2005; Lau et al., 2007; Lemon et al., 2003; Liu et al., 2002a; Shea and Rogers, 2002; Shukitt-Hale et al., 2007; Shukitt-Hale et al., 2008a; Shukitt-Hale et al., 2008b; Singh et al., 2008; Tchantchou et al., 2004). Studies in culture have provided additional evidence for neuroprotective efficacy of some of these constituents (Chan and Shea, 2009; Dhitavat et al., 2001; Joseph et al., 2007; Ortiz and Shea, 2004; Shea et al., 2002; Shea et al., 2003; Zhu et al., 2007). Still less is known regarding the impact of consumption of nuts or their constituents on age-related disorders of the peripheral nervous system, although antioxidants have been demonstrated beneficial in spinal and peripheral nerve development and following injury (Hayashi, 2009; Hoshida et al., 2008). Nutritional supplements were also an effective addition to treatment of spinal muscular atrophy (Narver et al., 2008).

Amyotrophic lateral sclerosis (ALS) is an age-related disorder characterized by a progressive loss of motor neurons, with eventual degeneration of muscles themselves, resulting in paralysis and death. The onset and progression of ALS varies tremendously among affected individuals. The full range of causative factors remain elusive despite decades of study, but oxidative stress is recognized as one underlying contributor to motor neuron degeneration (Barber et al., 2006; Bruijn et al., 2004; Dupuis et al., 2004; Strong, 2003). Aberrant function of Cu/Zn superoxide dismutase 1 (SOD-1) is associated with ALS (Julien and Mushynski, 1998; Rosen et al., 1993; Strong, 2003). SOD-1 is abundant within motor neurons, where it converts superoxide...
anions to hydrogen peroxide. Mutations in SOD-1 promote neurodegeneration by a gain of toxic function, which promotes increased reactive oxygen species (ROS) and widespread oxidative damage to proteins, nucleic acids and lipids. More than 100 different mutations have been described that span all exons (Bruijn et al., 2004). Mutations in SOD-1 account for only 20% of the familial cases of ALS, which themselves make up only 5-10% of the total cases, indicating that one or more additional factors is/are involved in the onset and/or progression of ALS. ALS may therefore represent a complex disorder that can arise from several stages along the way (Rothstein, 1996; Strong, 2003).

Transgenic mice expressing mutant SOD-1, in particular the G93A mutation, have been widely utilized to study the onset and progression of ALS. These mice display progressive motor deterioration commencing between the 3rd and 4th month of life, typically initiating in lower limbs, and eventually encompasses all limbs, leading to death between the 5th and 6th month of life (Gurney et al., 1994). In addition to overt neuromuscular compromise, these mice also display multiple biochemical and neurological pathological changes analogous to human ALS (Cozzolino et al., 2008; Hensley et al., 2006; Shibata, 2001) and as such represent a useful model system for development of therapeutic interventions (Turner and Talbot, 2008). These mice display upregulated compensatory antioxidant activity that is apparently insufficient to counteract the consequences of overexpression of human mutant SOD-1 (Mahoney et al., 2006). Antioxidant supplementation can delay the progression of decline in motor neuron function (Barber et al., 2006; Gurney et al., 1996; Jung et al., 2001; Liu et al., 2002b; Turner and Talbot, 2008). We examined herein whether or not dietary supplementation with pecans could affect the course of motor neuron pathology in this mouse model.

**MATERIALS AND METHODS**

**Mouse Strains and Diets:** Transgenic mice expressing G93A mutant human SOD-1 and corresponding controls expressing normal human SOD-1, (B6SJL-TgN (SOD1-G93A) 1 Gur and B6SJL-TgN (SOD1)2Gur, respectively, from Jackson Labs) were maintained on a standard, complete diet (AIN-76”; Purina/ TestDiets, Inc.; Table 1). Pecans (obtained from Young Pecan Shelling Company, Florence, SC) were ground into powder and mixed thoroughly within the diet powder during preparation by TestDiets, Inc. Consistent with prior studies, mice expressing normal human SOD-1 did not display any decline in motor neuron function over the course of 3 months when maintained on this complete diet (not shown). Groups of mice expressing G93A mutant human SOD-1 received the above diet without supplementation or supplemented with 0.01-2% pecans (based on wet weight of diet) commencing at 2 months of age. All mice were weighed weekly. Our Institutional Animal Care and Use Committee approved all procedures.

**Quantification of Neuromuscular Performance:** Mice were monitored for motor performance at the start of the above supplementation regimen by disease onset and progression using the “classical” criteria: disease onset was defined as a sharp, dramatic decline in ability to cling to a wire (referred to as the “wire cling” or “paw grip endurance” test) (Barneoud et al., 1997; Gurney et al., 1994; Suchy et al., 2009; Weydt et al., 2003). The maximum length of time scored was 60 sec. (i.e., if a mouse could cling for 60 sec, the test was halted (Suchy et al., 2009; Weydt et al., 2003). “Disease progression” was defined as death or the inability of a mouse to right itself within 30 sec when placed on its back; this time defined as “surrogate death” and scored with equal weight as actual death (Jung et al., 2001; Suchy et al., 2009; Weydt et al., 2003). Reported values represent the percentage of mice that retained the ability to right themselves within 30 sec versus the total number of mice at the start of the experiment. We also quantified motor performance according to a “Motor Function

<table>
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<th>Table 1. Composition of diet</th>
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<td><strong>Vitamins</strong></td>
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<td>Riboflavin (ppm)</td>
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<td>Niacin (ppm)</td>
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<td>Panthothenic Acid (ppm)</td>
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<td>Folic Acid (ppm)</td>
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<td>Pyridoxine (ppm)</td>
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<td>Biotin (ppm)</td>
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<td>Vitamin B12 (µg/kg)</td>
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<td>Choline Chloride (ppm)</td>
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<td>Iodine (ppm)</td>
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<td>Chromium (ppm)</td>
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<tr>
<td>Molybdenum (ppm)</td>
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<td>Selenium (ppm)</td>
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Pecans delay motor neuron decline in mice

Score Sheet,” modified from prior versions (Suchy et al., 2009; Weydt et al., 2005; Weydt et al., 2003). In this additional approach, mice were arbitrarily ranked from 0-4 (with 0 corresponding to no detectable motor deficits and 4 corresponding to death/surrogate death). Rankings of 1-3 provide intermediate classifications of mild, moderate or severe motor deficits as follows:

1: Mild deficit: abnormal gait with hind-limb tremors when suspended by the tail, but still mobile
2: Moderate deficit: bilateral hind-limb paraplegia, with mobility and ability to reach food
3: Severe deficit: bilateral hind-limb paralysis, unable to move freely within cage ± development of pressure lesions or urine scalding on ventral body wall. Note that urine scalding is a consequence of the animal being unable to move away from urine following urination rather than incontinence. For the motor score sheet, a score of 0 was considered as 100%, 1 as 75%, 2 as 50%, 3 as 25% and 4 as 0%.

Each mouse was scored individually in all tests, and the mean and standard error of the mean was calculated for all percentages. Scores between pecan-supplemented mice and non-supplemented mice were statistically compared at each time point for each test using Student’s \( t \) test; values were considered significant when \( p<0.05 \).

In preliminary experiments, statistically significant beneficial effects in neuromuscular performance were observed only for mice receiving a diet supplemented with 0.05% pecans; continued experiments therefore focused on this concentration for supplementation. For simplicity, we therefore present motor performance data only for non-supplemented mice and mice receiving a diet supplemented with 0.05% pecans. Based on an average dietary consumption of 3g/food/day (e.g., Rogers et al., 2004), each mouse therefore consumed an average of 1.5mg pecans/day. In accordance with these neuromuscular performance data, immunological analyses as described below were carried only for these groups 5 weeks after initiation of the supplementation regimen (at which time a statistical difference was observed between non-supplemented and pecan-supplemented mice (e.g., Fig. 1).

![Graphs from figure 1 showing supplementation with pecans delays early aspects of neuromuscular decline.](image-url)
**Motor neuron quantification:** Lumbar spinal cord segments from mice on each diet 5 weeks after initiation of the supplementation regimen were fixed in a mixture of 10% formalin, 10% acetic acid, and 80% ethanol for 4 hr, dehydrated in ethanol and embedded in paraffin. Sections (10μM) were deparaffinized, rehydrated, quenched with 3% H2O2, incubated in 5% horse serum in Tris-buffered saline (TBS) with 0.1% Triton-X 100 for 1 hr, then reacted overnight at 4°C with a monoclonal antibody (SMI-32) directed against non-phosphorylated neurofilament epitopes (Stemberg Monoclonals, MD) diluted 1:2000 in TBS containing 0.1% Triton X-100. Sections were washed 3x with TBS containing 0.1% Triton X-100, incubated with a biotinylated horse anti-mouse IgG for 2 hr and visualized with diaminobenzidine tetrahydrochloride (DAB) using the Vectastain ABC kit (Vector Laboratories, CA). Motor neurons are readily identified by their large SMI32 positive cell bodies (> 20µm) located in the ventral horn area of spinal cord transverse sections. Motor neurons were scored in a total of 47 sections derived from 3 mice maintained on the complete diet or the complete diet supplemented with 0.05% pecans.

Quantification of SOD-1 and glial fibrillary acidic protein (GFAP) Lumbar spinal cords from 3 mice on each diet 5 weeks after initiation of the supplementation regimen were sonicated in 10 volumes of 50mM Tris (pH 7.5) containing 150mM NaCl, 1% Triton X-100 and a protease Inhibitor cocktail (Roche). Homogenates were centrifuged at 20,000x g (4°C) for 15 min. The concentration of resulting lysates was determined by bicinchoninic acid protein assay (Thermo scientific). Normalized supernatants were electrophoresed on 4-12% SDS polyacrylamide gradient gels (Thermo scientific), and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 2.7 mM KCl, 137 mM NaCl containing 0.1% Tween; “TBS-T”) and probed with sheep anti-SOD1 (1:1,000; Calbiochem) and rabbit anti-GFAP (1:500; Dako USA) 1% non-fat dry milk in TBS-T overnight at 4°C. After 3 washes with TBS-T, the membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1 h. Immunoreactive species were detected using an enhanced chemiluminescence protocol (Thermo scientific). Images were captured using a Chemidoc imager (Syngene, UK) and quantified using NIH image.

**RESULTS**

Supplementation with pecans statistically delayed the first signs of decline in motor performance. Nonsupplemented mice displayed an initial decline in motor performance as ascertained by the motor function test between weeks 2 and 3 following initiation of the dietary regimen utilized herein (Fig. 1). By contrast, mice maintained on the pecan-supplemented diet did not display a decline until between weeks 4 and 5. Supplementation with pecans provided neuroprotection until week 10 following initiation of the dietary regimen, after which supplemented and non-supplemented mice displayed identical rates of decline (Fig. 1).

A decline in paw grip endurance for non-supplemented mice was first observed between weeks 6 and 7, and a decline in righting ability was first observed between weeks 9 and 10, with rapid and progressive continued decline in function in both of these tests; supplementation with pecans did not alter the onset or progression of decline in motor neuron function in either of these tests (Fig. 1). There was no significant weight difference between supplemented and non-supplemented mice; no decline in weight was observed in mice up to and including week 12 (Table 2), by which time motor neuron function had begun to decline significantly (Fig. 1), suggesting that all deaths resulted from presence of the G93A SOD-1 gene (Scott et al., 2008).

**FIGURE 2. Supplementation with pecans delays motor neuron loss.** A significant increase (p<0.05) in motor neurons were observed in anterior horn sections from lumbar spinal cord of mice receiving a diet supplemented with 0.05% pecans as compared to non-supplemented mice. Values represent mean (± standard error of the mean) number of motor neurons per section scored in 47 sections derived from 3 mice maintained for 5 weeks under each diet.

**TABLE 2. Changes in weight of mice during treatment.** All mice were weighed weekly. Values represent average weight of mice (weight in grams ± standard deviation) on non-supplemented and 0.05% pecan-supplemented diets at the indicated weeks. Note the lack of difference between non-supplemented and supplemented mice, and the lack of weight up to and including week 12, by which time mice had begun to undergo death/surrogate death. with pecans significantly delayed the decline in motor performance.

<table>
<thead>
<tr>
<th>Weeks on Diet</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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<tr>
<td>Non-supplemented</td>
<td>17.9 ± 2.4</td>
<td>18.2 ± 3.1</td>
<td>18.6 ± 2.8</td>
<td>19.5 ± 3.1</td>
<td>19.1 ± 3.1</td>
<td>20.3 ± 2.8</td>
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<tr>
<td>Supplemented with 0.05% pecans</td>
<td>18.9 ± 1.8</td>
<td>19.4 ± 1.9</td>
<td>20.1 ± 2.1</td>
<td>21.4 ± 2.6</td>
<td>19.8 ± 1.8</td>
<td>18.4 ± 1.4</td>
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Based on the above delay of decline in motor performance following supplementation with pecans, we quantified motor neurons within lumbar spinal sections in supplemented and nonsupplemented mice at week 5 after initiating supplementation. Consistent with the delay in neuromuscular performance (Fig. 1), we observed significantly more ($p<0.01$) motor neurons at 5 weeks in pecan-supplemented versus nonsupplemented mice (Fig. 2).

Homogenates of lumbar spinal cord at week 5 were subjected to immunoblot analyses for human SOD-1. These analyses confirmed equivalent expression of the transgene in both nonsupplemented and supplemented mice, but also demonstrated that that supplementation with pecans significantly ($p<0.05$) decreased oligomerization of SOD-1 (Fig. 3). The above homogenates were also examined for GFAP as an index of gliosis. Pecan supplementation statistically ($p<0.05$) decreased total GFAP levels in lumbar spinal cord, and eliminated GFAP breakdown products that can accompany ALS (Fig. 4) (Fujita et al., 1998).

**DISCUSSION**

Supplementation with pecans significantly delayed the earliest detectable aspects of motor neuron function, revealed by monitoring the onset of hind limb tremors. This transient delay was accompanied by a preservation of motor neuron number in lumbar spinal cord, along with a corresponding reduction in gliosis and aggregation of SOD-1, both of which are hallmarks of motor neuron decline in these mice and in ALS (Bruijn et al., 1998; Fujita et al., 1998; Johnston et al., 2000; Levine et al., 1999; Rakhit et al., 2004; Watanabe et al., 2001). Since over time motor function declined to equivalent levels in supplemented and nonsupplemented mice, we did not carry out continued analyses of biochemical parameters at later intervals. The timing of more severe aspects of neuromuscular decline as observed herein utilizing routine tests of motor function (paw grip endurance and righting ability) was consistent with that of prior studies, and was not altered by pecan supplementation. Nevertheless, we observed a 2-3 week delay in initial neuromuscular decline, along with a sustained degree of neuroprotection as late as 9-10 weeks of our feeding regimen (which corresponds to > 4 months of age). In accord with the average murine lifespan of 2.5-3 years versus that of humans, extrapolation of this delay to human ALS suggests that appropriate nutritional supplementation holds the promise of improving the quality of life during progression of this disease. It
should be noted that compounds demonstrating efficacy in ALS delay either the onset or progression of neuromuscular decline, but not both (Barneoud and Curet, 1999; Fornai et al., 2008; Grunfeld et al., 2007; Guo et al., 2003; Holzbaur et al., 2006; Jung et al., 2001; Park et al., 2007a; Wang and Zhang, 2005; Weydt et al., 2005; Zhang et al., 2008).

We observed neuroprotection with only one concentration of pecans. While concentrations lower than 0.05% of the total diet may simply not provide enough beneficial constituents, why 1-2% are not equally or more beneficial than 0.05% was not clarified. However, we previously observed a similar situation following dietary supplementation with apple juice concentrate in another mouse strain. In this prior study, however, we observed that even a doubling of apple juice compromised overall food intake, underscoring that supplementation must be accompanied by a complete and sufficient dietary regimen to provide neuroprotection (Rogers et al., 2004). We did not quantify food intake herein but suspect that increasing pecan concentration beyond the critical neuroprotective level may have compromised overall food consumption. It also remains possible that one or more constituents of pecans are neuroprotective only at the concentration achieved by dietary supplementation with pecans at 0.05% of the total diet weight as provided herein.

Nutritional requirements of key neuroprotectants may increase during aging (Drewnowski and Warren-Mears, 2001). Unfortunately, dietary quality often decreases with age even beneath normal conditions (Chernoff, 2001). However, key dietary interventions can modulate or delay age-related diseases (Everitt et al., 2006). Nutritional intake declines critically under conditions of motor neuron dysfunction (Rosenfeld and Ellis, 2008). A number of nutritional supplements have demonstrated a degree of clinical efficacy in ALS, including high doses of B vitamins, constituents of red wine and green tea (Esposito et al., 2000; Izumi and Kaji, 2007; Xu et al., 2006). Pyruvate, which provides both antioxidant activity and a source of energy, was also effective (Park et al., 2007b). It is debated as to whether oxidative stress is a primary or secondary feature of ALS (Agar and Durham, 2003). Nevertheless, since oxidative stress that accompanies ALS triggers multiple downstream deleterious consequences (Dupuis et al., 2004; Ito et al., 2008), antioxidant therapy remains an important approach. Notably, a combination of polyunsaturated fats and vitamin E, both of which are constituents of pecans, was beneficial (Veldink et al., 2007). Pecans, like many nuts, contain polyunsaturated fats (Ryan et al., 2006) and lower levels of low-density lipoprotein cholesterol under normal conditions. The findings herein support inclusion of pecans and/or other nuts as part of a comprehensive nutritional therapeutic approach that may augment pharmacological approaches.

ACKNOWLEDGMENT

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